University of Alberta

A Novel Deprenyl Analogue, N-methyl, N-propargyl-2-phenylethylamine (MPPE), Increases Neuronal Cell Survival in Thiamine Deficiency Encephalopathy

by

Eva Kwan ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Psychiatry

Edmonton, Alberta

Spring 2002

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-69722-3

Canada

University of Alberta

Library Release Form

Name of Author: Eva Kwan

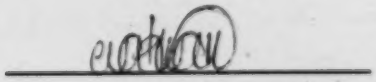
Title of Thesis: A Novel Deprenyl Analogue, N-methyl, N-propargyl-2-phenylethylamine (MPPE), Increases Neuronal Cell Survival in Thiamine Deficiency Encephalopathy

Degree: Master of Science

Year this Degree Granted: 2002

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.



Eva Kwan
14007 101 A avenue
Edmonton, Alberta
T5N 0L4

Date submitted: December 19, 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled A Novel Deprenyl Analogue, N-methyl, N-propargyl-2-phenylethylamine (MPPE), Increases Neuronal Cell Survival in Thiamine Deficiency Encephalopathy submitted by Eva Kwan in partial fulfillment of the requirements for the degree of Master of Science.

Dr. Kathryn G. Todd

Dr. Glen B. Baker

Dr. Ashfaq Shuaib

Date approved: December 18 2001

DEDICATION

This thesis is dedicated to Curtis for his unconditional love and support, and to Curran, for helping to put things into perspective.

ABSTRACT

Wernicke Encephalopathy (WE) is a neurological disorder attributable to thiamine deficiency (TD). Severe TD, in humans and animals, results in highly selective focal lesions with symmetrical distribution in the central nervous system. Anatomically, the brain regions consistently found to develop lesions include the mammillary bodies, thalamic nuclei, inferior colliculi, periaqueductal and periventricular regions, vestibular nuclei, and the cerebellum. Experimental TD in the rat provides a robust and reproducible model that allows investigations of mechanisms underlying both apoptotic and necrotic death.

(-)-Deprenyl (DEP), a selective monoamine oxidase B (MAO-B) inhibitor, has been used in the treatment of neurodegenerative disorders and to demonstrate neuroprotective and/or neurorescue properties in a variety of *ex vivo* and *in vitro* paradigms. It has been previously reported that daily administration of DEP reduces the amount of neuronal cell death that occurs in vulnerable brain areas of rats made thiamine deficient. Because the metabolites of DEP, e.g. amphetamine and methamphetamine, may have adverse behavioral effects, a novel DEP analogue, N, methyl, N-propargyl-2-phenylethylamine (MPPE), was synthesized that would not have amphetamine-like metabolites. In the present investigation, MPPE was assessed for: its neuroprotective potential using histology and immunohistochemistry; its effect on MAO activity; and its effect on levels of biogenic amines, their acid metabolites, and amino acids in the TD model of neuronal cell death.

ACKNOWLEDGEMENTS

I wish to thank my supervisors, Dr. Kathryn Todd and Dr. Glen Baker, for providing me with the opportunity to conduct this research, as well as Dr. Ashfaq Shuaib for serving as a member of my supervisory and examining committee. I also wish to acknowledge the Faculty of Medicine/Neurology, the Department of Psychiatry, and the George and Dorothy Davey Endowment for Brain and Injury Research for the financial assistance through my program.

Thank you to the team of the NRU, especially to Gail Rauw, for their help and expertise with lab and non-lab issues. Judith Altarejos, Emily Gordon, Rob Clements, Mia Biondo, and Stacie Fedick - I am eternally grateful for your friendship and insight.

To my amazing parents, Bill and Agnes Kwan, I thank you for the love, support, and encouragement that you have given me throughout my life. Dad – you are my hero. And finally, a great big thank you to Curtis - you have been a source of strength for me, and I could not have done this without you.

TABLE OF CONTENTS

Page

CHAPTER 1 Introduction

1.1. NEURONAL CELL DEATH.....1

1.2. THIAMINE DEFICIENCY ENCEPHALOPATHY.....2

1.2.1. Wernicke encephalopathy.....2

1.2.2. Experimental thiamine deficiency.....3

1.2.3. Pathophysiological mechanisms.....8

1.3. (-)-DEPRENYL (DEP).....11

1.3.1. Monoamine oxidase (MAO).....11

1.3.2. DEP use in psychiatry.....12

1.3.3. Neuroprotective/neurorescue effects.....13

1.3.4. Mechanisms of action.....14

1.4. N-METHYL,N-PROPARGYL-2-PHENYLETHYLAMINE (MPPE).18

1.5. THESIS OBJECTIVE AND RATIONALE.....20

CHAPTER 2 Materials and methods

2.1. MATERIALS.....22

2.1.1. List of chemicals and reagents used.....22

2.1.2. List of instrumentation and apparatus employed.....25

2.1.3. Animals.....27

2.2. METHODS.....27

2.2.1. Preparation of pyrithiamine.....27

2.2.2. Preparation of thiamine.....27

2.2.3. Preparation of DEP.....28

2.2.4. Preparation of MPPE.....28

2.2.5. Thiamine deficiency protocol.....28

2.2.5.1. Treatment groups.....29

2.2.5.2. Administration of drugs.....29

2.2.5.3. Reversal stage animals.....30

2.2.5.4. Symptomatic stage animals.....30

2.2.6. General sample handling.....31

2.2.6.1. Processing for sectioning.....31

2.2.6.2. Processing for biochemical analyses.....32

2.2.6. Nissl processing.....32

2.2.6.1. Cresyl violet protocol.....33

2.2.6.2. Neuronal cell count determination.....33

2.2.7. Immunohistochemistry.....34

2.2.7.1. Preparation of phosphate buffered saline (PBS).....34

2.2.7.2. Identification of astrocytes.....33

2.2.7.3. Microglia identification.....35

2.2.7.4. Quantification of immunoreactivity.....36

2.2.8. Monoamine oxidase (MAO) estimation.....36

2.2.8.1. Preparation of potassium phosphate buffer.....37

2.2.8.2. Radio-labeled substrates.....37

2.2.8.3. Preparation of ethyl acetate/toluene.....37

2.2.8.4. Protocol for samples.....37

2.2.9. High performance liquid chromatography (HPLC).....38

2.2.9.1. Determination of biogenic amine levels.....39

2.2.9.1.1. Mobile phase preparation.....39

2.2.9.1.2. Preparation of perchloric acid solution.....39

2.2.9.1.3. Protocol for samples.....39

2.2.9.2. Determination of amino acid levels.....40

2.2.9.2.1. Preparation of mobile phases.....40

2.2.9.2.2. Protocol for samples.....41

2.3. STATISTICAL ANALYSES.....42

CHAPTER 3 Results

3.1. BEHAVIOR.....43

3.2. HISTOLOGY AND IMMUNOHISTOCHEMISTRY.....43

3.2.1. Neuronal cell counts.....43

3.2.2. Immunoreactivity.....46

3.3. MAO ACTIVITY.....53

3.4. LEVELS OF BIOGENIC AMINES AND THEIR ACID METABOLITES.....55

3.5. LEVELS OF AMINO ACIDS 60

CHAPTER 4 Discussion

4.1. SUMMARY AND IMPLICATIONS.....64

4.2. LIMITATIONS AND FUTURE DIRECTIONS.....70

4.3. CONCLUSIONS.....74

CHAPTER 5 Bibliography

5.1. BIBLIOGRAPHY.....75

LIST OF TABLES

Page

Table 2.1. Chemical and reagent list.....22

Table 2.2. Instrumentation and apparatus list.....25

Table 3.1. Neuronal cell counts.....45

Table 3.2. GFAP intensity scores.....47

Table 3.3. ED1 intensity scores.....48

LIST OF FIGURES

Page

Figure 1.1. Chemical structures of thiamine and pyrithiamine.....5

Figure 1.1. Behavioral manifestations in experimental TD.....6

Figure 1.2. Pathophysiological mechanisms involved in TD.....9

Figure 1.3. Chemical structures of DEP metabolites.....17

Figure 1.4. Chemical structures of DEP and MPPE.....19

Figure 3.1. Graphical representation of neuronal cell counts.....45

Figure 3.2. Graphical representation of GFAP immunoreactivity.....47

Figure 3.3. Graphical representation of ED1 immunoreactivity.....48

Figure 3.4. Photomicrographs of histology and immunohistochemistry...50

Figure 3.5. Photomicrographs of GFAP and ED1 immunoreactivity.....52

Figure 3.6. MAO activity.....54

Figure 3.7. Chromatograms of biogenic amines and their acid metabolites.....57

Figure 3.8. Levels of DA and 5-HT.....58

Figure 3.9. Levels of DOPAC and HVA.....59

Figure 3.10. Chromatograms of amino acids.....61

Figure 3.11. Levels of GLU, ASP, and ALA.....62

Figure 3.12. Levels of GLN and TAUR.....63

LIST OF SYMBOLS AND ABBREVIATIONS

%	percent
x	multiplied by
=	equals
>	more than
<	less than
±	plus or minus
↑	increased
↓	decreased
°C	degrees Celsius
m.w.	molecular weight
e.g.	for example
et al.	and colleagues
ng	nanogram
µg	microgram
g	gram
mg	milligram
kg	kilogram
µl	microliter
ml	milliliter
min	minute
h	hour

µm	micrometer
mm	millimeter
rpm	revolutions per minute
µCi	microCurie
µmol	micromolar
mM	millimolar
M	molar
N	normal
v/v	volume to volume
TD	thiamine deficiency
TDP	thiamine diphosphate
CNS	central nervous system
BBB	blood-brain barrier
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
DNA	deoxyribonucleic acid
TK	transketolase
PDH	pyruvate dehydrogenase
αKGDH	*alpha* ketoglutarate dehydrogenase
NMDA	N-methyl-D-aspartate
ROS	reactive oxygen species
NOS	nitric oxide synthase
NO	nitric oxide

IEG	immediate early genes
SOD	superoxide dismutase
Ca^{2+}	intracellular calcium levels
MPP+	1-methyl-4-1,2,3,6-tetrahydropyridinium ion
DSP-4	*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine
GFAP	glial fibrillary acidic protein
DEP	(-)-deprenyl
dmDEP	N-desmethyldeprenyl
METH	methamphetamine
AM	amphetamine
MAO	monoamine oxidase
NA	noradrenaline
5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	homovanillic acid
PEA	2-phenylethylamine
CYP	cytochrome P450
MPPE	N-methyl, N-propargyl-2-phenylethylamine
NaCl	sodium chloride
KCl	potassium chloride
Na_2HPO_4	sodium phosphate, dibasic

KH_2PO_4	potassium phosphate, monobasic
HCl	hydrochloric acid
KOH	potassium hydroxide
H_2O_2	hydrogen peroxide
DAB	3,3'-diaminobenzidine tetrahydrochloride
$HClO_4$	perchloric acid
EDTA	disodium ethylenediaminetetraacetate
NaH_2PO_4	sodium phosphate, monobasic
CH_3CN	acetonitrile
SOS	sodium octyl sulfate
OPA	o-phthaldialdehyde
MeOH	methanol
THF	tetrahydrofuran
N_2	nitrogen gas
HPLC	high performance liquid chromatography
n	group sample size
CON	control
VEH	saline vehicle
TD/VEH	pyrithiamine and saline
TD/DEP	pyrithiamine and deprenyl
TD/MPPE	pyrithiamine and N-methyl, N-propargyl-2-phenylethylamine
CON/VEH	thiamine and saline
CON/DEP	thiamine and deprenyl

CON/MPPE	thiamine and N-methyl, N-propargyl-2-phenylethylamine
s.c.	subcutaneous
i.p.	intraperitoneal
THIA	thiamine
PYR	pyrithiamine
PBS	phosphate buffered saline
SEM	standard error of the mean
GLU	glutamate
ASP	aspartate
ALA	alanine
GLY	glycine
GLN	glutamine
TAUR	taurine
GABA	γ-aminobutyric acid
SER	serine
ASN	asparagine

CHAPTER 1 Introduction

1.1. NEURONAL CELL DEATH

In the central nervous system (CNS), there are two types of cells: nerve cells (neurons) and glial cells. During the development of the CNS, neuronal cell death occurs in normal cell differentiation (Oppenheim, 1991). It also contributes to many disorders of the brain, including stroke, brain trauma, and neurodegenerative diseases (Hutchins and Barger, 1998). There are two main categories of neuronal cell death: apoptosis, or programmed cell death, and necrosis. Apoptosis is an active process, requiring RNA and protein synthesis (Hutchins and Barger, 1998). Volume loss, membrane blebbing, nuclear condensation, and DNA fragmentation characterize apoptotic cells (Dragovich et al., 1998; Hutchins and Barger, 1998). In contrast, neurons undergoing necrosis swell and lyse in a passive process (Dragovich et al., 1998; Hutchins and Barger, 1998). There are three criteria that have been used to identify neuronal apoptosis: DNA fragmentation, cell shrinkage and the condensation of chromatin, and a requirement for new protein synthesis (Tatton and Chalmers-Redmen, 1996). The standard for determining whether cell death is apoptotic or necrotic is based on differences in morphology (Darzynkiewicz et al., 1998). Despite the different mechanisms and characteristic morphology of each type of cell death, there is not always a clear distinction between the two. For example, both types of cell death can occur in close proximity in the same tissue. One

useful determinant between these modes of death is the implication that the death of a neuron, or group of neurons, has for its neighbors. Apoptotic death is tidy and controlled, involving single cells or clusters of cells, whereas necrotic death is rapid and abrupt, involving the leakage of cellular contents, and due to the degradative action of enzymes (Leist and Nicotera, 1998; Cotran et al., 1989). Classically, apoptosis is defined as a type of cell death that does not induce inflammation as necrotic death does; however, in pathological conditions, induction of inflammation by Fas ligand, a well-characterized apoptotic inducer, has been demonstrated (Miwa et al., 1998). Apoptosis proceeds through an ordered program of events that is regulated by the cell, and the importance of this cascade is highlighted by the likelihood that disruption or failure of apoptosis may result in the development of cancer (Alnemri, 1997). Thus, apoptotic research has developed to become one of the largest areas in biological research due to its association with several human diseases ranging from cancer to neurodegenerative diseases.

1.2. THIAMINE DEFICIENCY ENCEPHALOPATHY

1.2.1. Wernicke encephalopathy

The model that was used in the project described in this thesis to study neuronal cell death is thiamine deficiency encephalopathy. Thiamine (vitamin B1) deficiency is becoming more frequently observed in a variety of disorders where the causative factor is malnourishment.

These disorders include anorexia nervosa, gastrointestinal disease, chronic alcoholism, and conditions causing persistent vomiting or requiring dialysis. In humans, thiamine deficiency (TD) is the cause of the CNS disorder Wernicke encephalopathy (Troncoso et al., 1981). The neurological syndrome associated with this disorder consists primarily of a confusional state, ophthalmoplegia, ataxia, and nystagmus. Anatomically, severe TD leads to symmetrical lesions of a variety of brain nuclei, including the thalamus, mammillary bodies, colliculi, and vestibular nuclei. Both necrotic and apoptotic cell death have been shown to occur in TD (Bettendorff et al., 1997; Matshushima et al., 1997). The use of this model is relevant to many human conditions with neuronal cell death as a common factor, such as ischemia, trauma, and degenerative disorders. Therefore, results produced from using this model to study the events that occur in neuronal cell death are pertinent to other disorders of the CNS.

1.2.2. Experimental thiamine deficiency

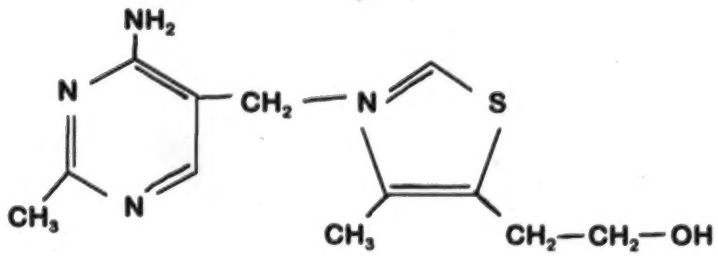
The neurological symptoms and selective, focal lesions observed in the human condition of Wernicke encephalopathy are recapitulated in the experimental model of TD. The neuronal cell death induced using this model is gradually evolving, with significant cell death occurring after 14 – 15 days of TD. This time course enables more discrete analysis of the sequence of events that occur during apoptosis and necrosis in the vulnerable brain regions, in contrast to other models of cell death such as

ischemia, where the insult and damage occur over a shorter period of time (e.g. minutes). In the experimental model, TD is induced by daily injections of the centrally active thiamine anti-metabolite, pyrithiamine (see Figure 1.1.) (Heroux and Butterworth, 1995). This compound crosses the blood-brain barrier (BBB) to rapidly deplete thiamine stores by inhibiting pyrophosphokinase, an enzyme that converts thiamine to its diphosphate ester (TDP) (Rindi and Perri, 1961), as well as inhibiting thiamine transport into brain tissue (Iwashima et al., 1976). Administration of this compound, in combination with a thiamine deficient diet, results in a robust and reproducible rat model of TD.

The behavioral manifestations observed in animals are correlated with the severity of TD (see Figure 1.2.). As thiamine status decreases, anorexia and weight loss are observed, followed by nystagmus and ataxia. Hypothermia is common, and grooming behavior declines over time. The loss of righting reflex, which is the rat's reflex to right itself when in a prone position, is indicative of neurological damage and severe TD. If allowed to progress, seizures, coma, and death will follow.

Figure 1.1. Chemical structures of thiamine and pyrithiamine

thiamine



pyrithiamine

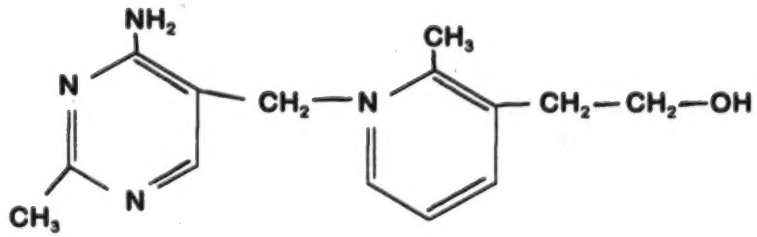
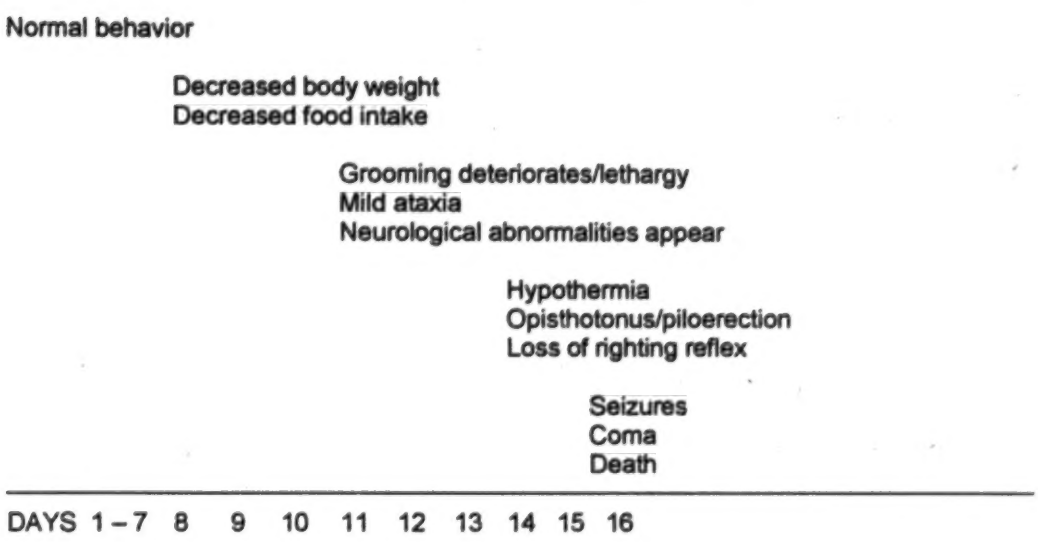


Figure 1.2. Behavioral manifestations in experimental TD

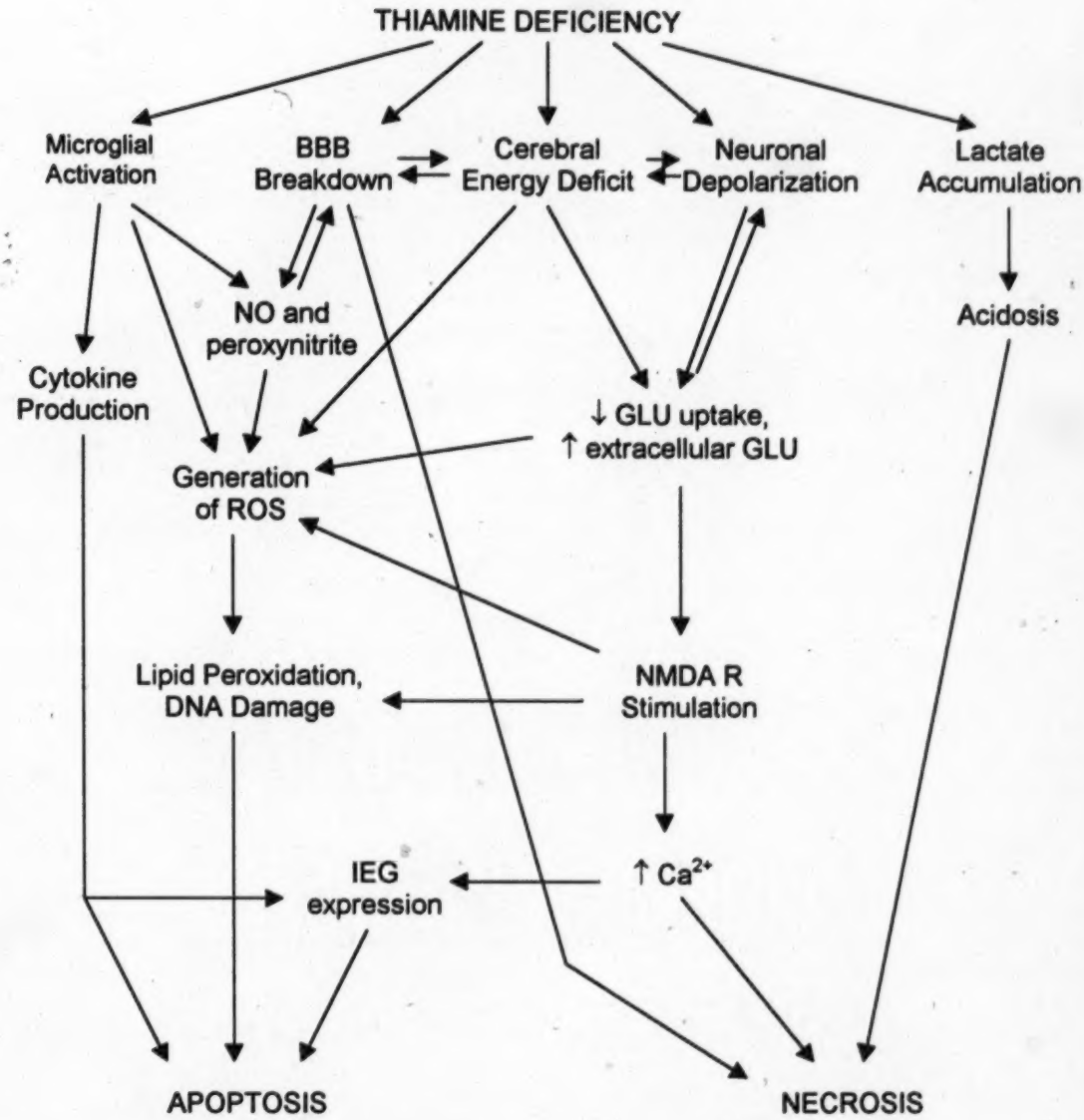


The distribution of thiamine is fairly even throughout the brain, averaging 13 μg/g dry weight (Cooper and Pincus, 1979). The half-life of thiamine is between 33 and 36 h in brain, heart, and liver (Sen and Cooper, 1976). It is estimated that 80% of thiamine in the body is in the form of TDP (Cooper and Pincus, 1979). TDP serves as a cofactor for three key enzymes participating in energy metabolism: transketolase (TK), pyruvate dehydrogenase (PDH), and *alpha* ketoglutarate dehydrogenase (αKGDH). If thiamine is given at the early stages of TD, the neurological symptoms are readily reversible, leading to the hypothesis of a 'biochemical lesion' (Peters, 1936). Investigations into this hypothesis reported that under conditions of TD, αKGDH and TK activities are significantly decreased (Gibson et al., 1984; Butterworth et al., 1986; Sheu et al., 1998), while PDH activity remains unchanged (Gibson et al., 1984; Munujos et al., 1996). Furthermore, the reversal of thiamine status restores αKGDH activity, but TK activity remains depressed (Gibson et al., 1984; Butterworth et al., 1986). However, the activities of these enzymes were altered in both vulnerable and non-vulnerable regions (Sheu et al., 1998; Giguere and Butterworth, 1987). Thus, the extensive examinations in the area of TDP-dependent enzymes have indicated that the 'biochemical lesion' hypothesis does not account for the selective nature of neuronal cell death seen in TD.

1.2.3. Pathophysiological mechanisms

Pathophysiological mechanisms that have been implicated in neuronal cell death induced by TD are numerous and interrelated (see Figure 1.3.). Some of the factors involved include impaired cellular energy metabolism (Aikawa et al., 1984), focal lactic acidosis (Munujos et al., 1996), NMDA receptor-mediated excitotoxicity (Hazell et al., 1998; Todd & Butterworth, 1998b), and increased expression of immediate early genes involved in apoptosis (Hazell et al., 1998). However, these influences have been shown to occur in the later stages of TD (day 12-13), when neurological symptoms are present and neuronal cell death has already been evidenced (Todd & Butterworth, 1998b, 1999). The timing of these factors suggests that they are not associated with reversible symptoms, but are likely involved in the structural damage observed in TD. Events that occur before any evidence of neuronal cell death or neurological symptoms induced by TD include microglial activation (day 8), the disruption of the BBB (day 10), and nitric oxide synthase (NOS) induction (day 10) (Todd and Butterworth, 1999; Calingasan et al., 1998). These early events implicate the involvement of the innate immune system of the brain, as well as the involvement of oxidative stress in the pathogenesis of lesions observed in TD.

Figure 1.3. Pathophysiological mechanisms involved in TD



BBB, blood-brain barrier; NO, nitric oxide; GLU, glutamate; ROS, reactive oxygen species; DNA, deoxyribonucleic acid; NMDA R, N-methyl-D-aspartate receptor; IEG, immediate early gene; Ca^{2+}, intracellular calcium.

The earliest immunohistochemical finding in TD is the activation of microglial cells (Todd and Butterworth, 1999). Microglia are the principal immune cells in the CNS, comprising between 5-15% of its cells (Carson and Sutcliffe, 1999). Like other immune cells, microglia have a dual function: to protect the CNS and to amplify inflammatory effects, which mediate cellular degeneration (Carson and Sutcliffe, 1999). Activated microglia migrate to the site of injury where they may proliferate, function as scavengers and antigen-presenting cells, and produce many physiologically active substances (Gonzalez-Scarano and Baltuch, 1999; Nakajima and Kohsaka, 1998), such as reactive oxygen species (ROS; Bañati et al., 1993). ROS can cause peroxidation of lipid membranes, resulting in neuronal rupture and death. Furthermore, oxidative stress leading to the formation of ROS has been associated with apoptosis (Wyllie et al., 1980). Confirming the involvement of ROS and oxidative stress in TD-induced cerebral damage, there are reports of increased ROS (Langlais et al., 1997). In addition, increased immunostaining for the free radical metabolizing enzyme superoxide dismutase (SOD; Todd and Butterworth, 1997), the antioxidant ferritin (Calingasan et al., 1998), and the oxidative stress marker heme oxygenase 1 (Calingasan et al., 1999) have been demonstrated in regions vulnerable to TD-induced neuronal cell death. Furthermore, Calingasan and colleagues (1998) demonstrated increases in immunoreactivity for the free-radical producing enzyme NOS and for peroxynitrite in these regions of TD brains. Nitric oxide (NO) has

important physiological functions in the nervous system, but also is potentially toxic: it can react with superoxide to produce the free radical peroxynitrite, and it affects the permeability of the BBB (Au et al., 1985).

In TD, the source of free radical production is unknown. One likely source is microglial cells, which have been shown to mediate neuronal cell injury through NO (Chao et al., 1992). As well, microglial cells have been shown to release iron on approximately day 10 of the TD protocol, which exacerbates the production of free radicals (Calingasan et al., 1998). Besides microglia, the seizures that occur in severely deficient animals can also elevate ROS production through NMDA receptors (Langlais et al., 1997). Taken together, these studies suggest that the impaired oxidative metabolism caused by TD is further exacerbated by the ROS production that may lead to the structural damage observed in TD brains.

1.3. (-)-DEPRENYL (DEP)

1.3.1. Monoamine oxidase (MAO)

Monoamine oxidase (MAO) catalyzes the oxidative deamination of monoamines, including neurotransmitters amines such as noradrenaline (NA), dopamine (DA), and 5-hydroxytryptamine (5-HT; serotonin). MAO exists in two forms that are differentially distributed: MAO-A is found primarily in neurons and MAO-B is found principally in non-neural cells, particularly in astrocytes (Tatton and Chalmers-Redman, 1996). Both isoenzymes have 70% amino acid identity and are encoded by two

different genes (Bach et al., 1988; Grimsby et al., 1991). In rodent brain, the ratio of MAO-A to B is approximately 1:1 (Green et al., 1977) while in human brain, the ratio is approximately 1:4 (Youdim and Finberg, 1991). MAO-A preferentially metabolizes 5HT and NA, while MAO-B preferentially deaminates 2-phenylethylamine (PEA) (Yu, 1986); several other biogenic amines are substrates for both MAO isoenzymes, such as DA. In rodents, DA is a substrate for both isoenzymes with equal affinity (Green et al., 1977). In primates, it has been demonstrated that there are regional differences with respect to which isoenzyme preferentially deaminates DA (Lakshmana et al., 1998). For example, in the hypothalamus and caudate nucleus, DA is metabolized preferentially by MAO-B; however, in motor cortex and hippocampus, each enzyme form metabolizes DA equally well (Lakshmana et al., 1998).

1.3.2. DEP use in psychiatry

(-)-Deprenyl (DEP) is a selective, irreversible MAO-B inhibitor at low concentrations, but it also inhibits MAO-A at higher concentrations or with repeated use (Gerlach et al., 1992). Because of the effects on MAO-A, the use of DEP in psychiatry is limited by the potential for adverse clinical side effects. MAO-A catalyzes the metabolism of tyramine in foods, and when its activity is compromised, tyramine-containing foods can cause a potentially serious condition known as the "cheese effect" which can result in severe hypertension (Blackwell et al., 1967). MAO-B

inhibition by DEP results in a decrease in dopamine (DA) catabolism and an increase in endogenous levels of PEA (Paterson et al., 1995). Clinically, DEP treatment has been reported to delay neurological deficits in Parkinson's disease (Parkinson Study Group, 1989, 1993). Interestingly, DEP has also been shown to attenuate the cognitive decline observed in Alzheimer's disease (Sunderland *et al.*, 1987; Filip and Kolibas, 1999). Possibilities for these effects on the neurodegenerative disorders include neuroprotective and/or neurorescue properties of DEP.

1.3.3. Neuroprotective/neurorescue effects

DEP treatment has been reported in *ex vivo* animal studies to increase neuronal survival after hypoxic/ischemic insult (Paterson *et al.*, 1996), mechanical injury (Salo and Tatton, 1992), and treatment with biochemical neurotoxins such as *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) (Finnegan *et al.*, 1990; Tatton and Greenwood, 1991; Zhang *et al.*, 1995). Treatment effects observed in the *ex vivo* studies are also demonstrated in *in vitro* experiments. For example, DEP treatment has been shown to protect neurons against damage due to neurotoxins such as N-methyl-D-aspartate and kainic acid (Shimazu et al., 1999; Gelowitz and Paterson, 1999), hypoxia (Xu et al., 1999), and trophic factor withdrawal (Tatton et al., 1994; Xu et al., 1999). These models showed that the increased neuronal cell survival observed following DEP treatment was independent of its MAO-B inhibition. For

example, using a dopaminergic cell line that does not contain MAO-B activity, Le *et al.* (1997) demonstrated that DEP treatment reduced neuronal cell death induced by administration of the DA neurotoxin 1-methyl-4-1,2,3,6-tetrahydropyridine ion (MPP+). All of these findings support a neuroprotective and/or neurorescue effect of DEP, independent of its ability to inhibit MAO-B.

DEP treatment has been demonstrated to protect against neuronal cell death due to TD (Todd and Butterworth, 1998a). As the regions that are vulnerable to TD contain fibers that are dopaminergic, noradrenergic, glutamatergic, and cholinergic, it is likely that the effects of DEP are not limited to monoamine-containing systems. Importantly, apoptosis is involved in a number of the models (e.g. axotomy and kainic acid-induced excitotoxicity), and DEP treatment reduced the neuronal death observed in those models. Thus, if apoptotic neuronal death is involved in human neurodegenerative diseases, then DEP or DEP analogues may have a role in their treatment.

1.3.4. Mechanisms of action

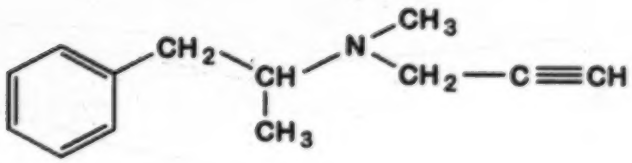
MAO-B inhibition results in a decrease in DA metabolism and an increase in endogenous levels of PEA (Paterson et al., 1995). As well, DEP can alter DA uptake (Fang and Yu, 1994). Other than its effects on MAO-B inhibition, several consequences of DEP treatment that may contribute to its neuroprotective/neurorescue effects have been reported.

DEP has been shown to prevent oxidative stress generated by DA metabolism by decreasing the production of the hydrogen peroxide peroxide by-product, a toxic intermediate oxygen species (Cohen and Spina, 1989). DEP also induces an increase in activity of SOD, an enzyme involved in defense against superoxide radicals (Carrillo et al., 1992; Thiffault et al., 1997). In addition, DEP treatment has been shown to alter glial fibrillary acidic protein (GFAP) immunoreactivity (Li et al., 1993; Revuelta et al., 1997) and to interact with glyceraldehyde-3-phosphate dehydrogenase (Kragten et al., 1998). It has been demonstrated that DEP induces increases in gene expression for neurotrophin ciliary neurotrophic factor (Seniuk *et al.*, 1994) as well as for basic fibroblast growth factor (Riva et al., 1997). Increases in mRNA levels of copper, zinc-dependent SOD (SOD-1) (Li *et al.*, 1998) and glial cell line-derived neurotrophic factor (Tang et al., 1998) have also been observed with DEP treatment. Furthermore, it was demonstrated that DEP treatment down-regulated expression of c-jun (Xu et al., 1999), which encodes an inducible transcription factor and contributes directly to programmed cell death (Dragunow and Preston, 1995). Thus, DEP may protect against neuronal cell death by preventing free radical formation, increasing antioxidant defenses, and altering gene expression (Tatton and Chalmers-Redman, 1996). However, the relative contribution of each of these factors to the neuroprotective/neurorescue effects of DEP treatment remains unknown. DEP increases neuronal survival in a variety of models

using different assaults and different cells, suggesting that DEP prevents cell death by interfering with an apoptotic pathway (Tatton et al., 1994; Xu et al., 1999).

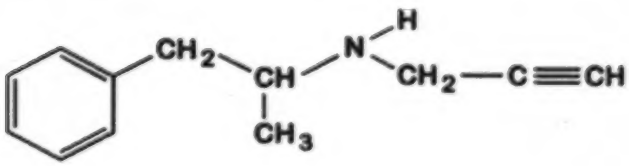
In the body, DEP is metabolized to N-desmethylDEP (dmDEP), amphetamine (AM) and methamphetamine (METH) (Reynolds *et al.*, 1978; Yoshida *et al.*, 1986), which are all pharmacologically active, albeit less active than the corresponding (+) forms with regard to CNS stimulant properties in the case of AM and METH. Reports in the literature suggest that all three of these metabolites (see figure 1.4. for structures) may have clinical significance with respect to the neuronal rescue and/or neuroprotective effects of their parent compound, DEP (Tatton and Chalmers-Redman, 1996; Mytilineou *et al.*, 1998; Stumm *et al.*, 1999). For example, dmDEP has been shown to possess neuroprotective properties in cultured mesencephalic neurons (Mytilineou et al., 1998). In contrast to the effects of dmDEP, both AM and METH have been demonstrated using *in vitro* and *ex vivo* studies to be detrimental to neuronal health and survival as well as hold a high abuse potential (Ricaurte *et al.*, 1985; Stumm *et al.*, 1999). Although controversy exists regarding whether the metabolites of DEP antagonize or promote neuroprotective effects (Tatton and Chalmers-Redman, 1996), compounds without potential AM-like properties are of interest as possible medications for the treatment of neurodegenerative disorders.

Figure 1.4. Chemical structures of DEP metabolites.

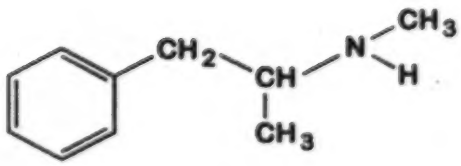


DEPRENYL

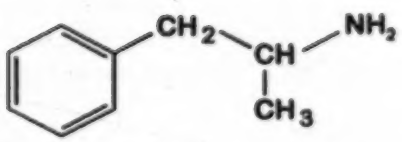
N-desmethyldeprenyl



methamphetamine



amphetamine

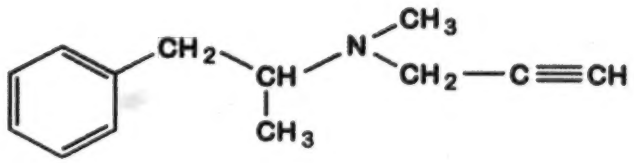


1.4. N-METHYL, N-PROPARGYL-2-PHENYLETHYLAMINE (MPPE)

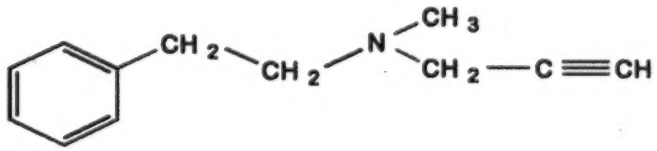
A novel DEP analogue that is not metabolized to AM or METH was synthesized by CV Technologies (Edmonton, Alberta). The chemical structure of the drug, N-methyl, N-propargyl-2-phenylethylamine (MPPE), compared to that of DEP, is shown in figure 1.5.

Figure 1.5. Chemical structures of DEP and MPPE

DEP



MPPE



1.5. THESIS OBJECTIVE AND RATIONALE

It has been well documented in the literature by numerous laboratories using a variety of differing *in vivo* paradigms and cultured cells that DEP possesses neurorescue and/or neuroprotective properties. Its use as adjunctive therapy to slow the progression of neurodegenerative diseases provides the impetus for the development of novel drugs that may be more efficacious in protecting or rescuing neurons, have a more desirable side effect profile, as well as provide insight into the mechanisms of action of DEP. DEP is rapidly absorbed and metabolized to dmDEP, METH, and AM by cytochrome P450 (CYP) enzymes in the liver (Heinonen et al., 1994), and all are pharmacologically active. A novel DEP analogue that does not produce AM metabolites has been synthesized and was examined for its neuroprotective potential. It has previously been shown that DEP treatment protects against neuronal cell loss due to TD (Todd and Butterworth, 1998a). Thus, the focus of the research described in this thesis was to evaluate the potential of MPPE to reduce or prevent brain cell death induced by TD in comparison to DEP. The hypotheses that were tested include: (1) MPPE would increase neuronal survival in TD encephalopathy; (2) MPPE is an MAO-B inhibitor; (3) MPPE would alter levels of biogenic amines, their acid metabolites, and amino acids similarly to that of DEP. To this end, thorough examinations of brain sections using histology and immunohistochemistry were performed to determine the effects of MPPE and DEP on neuronal

survival. In addition, a comprehensive neurochemical investigation of the effects of MPPE on activity of MAO and on levels of biogenic amines and amino acids of interest to brain function in comparison to the effects of DEP on these same measures was undertaken.

CHAPTER 2 Materials and methods

2.1. MATERIALS

2.1.1. List of chemicals and reagents used

Table 2.1. Chemical and reagent list

Chemical/reagent	Supplier
Pyrithiamine hydrobromide	Sigma Chemical Co.
Thiamine hydrochloride	Sigma Chemical Co.
Deprenyl (DEP)	Research Biochemicals Inc.
N-methyl, N-propargyl-2-phenylethylamine (MPPE)	CV Technologies
Thiamine deficient diet	ICN Nutritional Biochemical
10% phosphate buffered formalin	Fisher Scientific
Isopentane	Fisher Scientific
Xylene	Fisher Scientific
Ethanol	Biochemical stores
Cresyl violet	Fisher Scientific
Permount®	Fisher Scientific
Sodium chloride (NaCl)	Fisher Scientific
Potassium chloride (KCl)	Fisher Scientific
Sodium phosphate, dibasic (Na_2HPO_4)	Fisher Scientific
Potassium phosphate, monobasic (KH_2PO_4)	Fisher Scientific
Hydrochloric acid (HCl)	Fisher Scientific
Potassium hydroxide (KOH)	Fisher Scientific
Hydrogen peroxide (H_2O_2)	Fisher Scientific
Proteinase K	DAKO

Table 2.1. Chemical and reagent list (con't)

Chemical/reagent	Supplier
Universal blocker	DAKO
Glial fibrillary acidic protein	DAKO
Rabbit anti-cow immunoglobulin	DAKO
ED1	Serotec
Anti-mouse immunoglobulin	DAKO
Avidin-biotin complex	DAKO
3,3'-diaminobenzidine tetrahydrochloride (DAB)	Sigma Chemical Co.
Immunopen	Vector Laboratories
Ethyl acetate	Fisher Scientific
Toluene	Fisher Scientific
5-Hydroxy-[G-^{3}H]-tryptamine creatinine sulphate	NEN™ Life Science Products
2-Phenyl-[1-^{14}C]-ethylamine hydrochloride	NEN™ Life Science Products
Glacial acetic acid	Fisher Scientific
Ready Safe® liquid scintillation cocktail	Beckman Couter
Perchloric acid ($HClO_4$)	Fisher Scientific
disodium ethylenediaminetetra-acetate (EDTA)	Fisher Scientific
Sodium phosphate, monobasic (NaH_2PO_4)	Fisher Scientific
Acetonitrile (CH_3CN)	Fisher Scientific
sodium octyl sulfate (SOS)	Fisher Scientific
o-Phosphoric acid	Fisher Scientific

Table 2.1. Chemical and reagent list (con't)

Chemical/reagent	Supplier
Noradrenaline (NA)	Sigma Chemical Co.
Dopamine (DA)	Sigma Chemical Co.
3,4-Dihydroxyphenylacetic acid (DOPAC)	Sigma Chemical Co.
5-Hydroxytryptamine (5-HT)	Sigma Chemical Co.
5-Hydroxyindoleacetic acid (5-HIAA)	Sigma Chemical Co.
Homovanillic acid (HVA)	Sigma Chemical Co.
Methanol (MeOH)	Fisher Scientific
Tetrahydrofuran (THF)	Fisher Scientific
o-Phthaldialdehyde (OPA)	Pierce
Aspartate (ASP)	Sigma Chemical Co.
Glutamate (GLU)	Sigma Chemical Co.
Asparagine (ASN)	Sigma Chemical Co.
Serine (SER)	Sigma Chemical Co.
Glycine (GLY)	Sigma Chemical Co.
Glutamine (GLN)	Sigma Chemical Co.
Taurine (TAUR)	Sigma Chemical Co.
β-Alanine (β-ALA)	Sigma Chemical Co.
Alanine (ALA)	Sigma Chemical Co.
γ-Aminobutyric acid (GABA)	Sigma Chemical Co.

2.1.2. List of instrumentation and apparatus employed

Table 2.2. Instrumentation and apparatus list.

Instrument/apparatus	Supplier
AE 160 (weighing scale)	Mettler
27½ gauge needles	Becton-Dickinson
1 mL syringes	Becton-Dickinson
Microtome 0325R	Shandon Scientific Ltd.
Fisherbrand Superfrost*/Plus (microscope) slides	Fisher Scientific
Fisherfinest Premium cover glass	Fisher Scientific
Eclipse E600 (microscope)	Nikon
Tri-R Stir-R S63C (tissue homogenizer)	TriR Instruments, Inc.
Thermolyne Maxi Mix Vortex	Sybron/Thermolyne Instruments
Sorvall T6000B (centrifuge)	DuPont Instruments
LS 6000SC (scintillation counter)	Beckman Couter
Microcentrifuge	MSE
Waters μBondapak C18 column (pre-column)	Waters
Spherisorb ODS2 250 x 3.2mm (column for biogenic amines)	Phenomenex
Waters 460 electrochemical detector (for biogenic amines)	Waters
Waters spherisorb ODS2, C18 4.6.x 250 mm (column for amino acids)	Waters
Waters Alliance 2690 XE system (pump and sample management)	Waters

Table 2.2. Instrumentation and apparatus list (con't).

Instrument/apparatus	Supplier
Waters 474 fluorescence detector (for amino acids)	Waters
Millenium^{32} 3.05.01 (chromatography manager)	Waters
GraphPad™ Prism 3.0	Prism

2.1.3. Animals

For all studies described, male Sprague-Dawley rats initially weighing 200-250 g (BioScience Animal Services, Ellerslie, Alberta, Canada) were used. The animals were housed two per cage and maintained under constant ambient conditions of 20°C at 50% humidity with a 12h light-dark cycle. Upon arrival at our animal housing facility, rats were acclimatized to their new environment, were handled daily for 3 days, and provided with free access to regular food and water. All animal procedures were in accordance with the regulations of the Canadian Council on Animal Care and were approved by the Health Sciences Animal Welfare Committee of the University of Alberta.

2.2. METHODS

2.2.1. Preparation of pyrithiamine

Pyrithiamine hydrobromide (m.w.=420.1) was dissolved in saline and was made to a concentration of 10 mg in 40 ml saline in the dark. Aliquots of 7 ml were made and kept at -20°C until needed.

2.2.2. Preparation of thiamine

Thiamine hydrochloride (m.w.=337.3) was dissolved in saline to produce a concentration of 10 mg in 200 ml saline in the dark. Aliquots of 10 ml were made and kept at -20°C until required.

2.2.3. Preparation of DEP

DEP (m.w.=223.75) was dissolved in saline so that the final concentration was 1 mg per 1 ml saline. Fresh solutions of DEP were prepared each week and kept at 4°C until required.

2.2.4. Preparation of MPPE

MPPE (m.w.=209.72) was dissolved to give an almost equimolar concentration to DEP in saline (0.9 mg per 1 ml saline). Fresh solutions of MPPE were prepared each week and kept at 4°C until required.

2.2.5. Thiamine deficiency protocol

Thiamine deficiency (TD) was induced in rats according to previously published protocols (Todd and Butterworth, 1998a, 1998b, 1999). Rats assigned to the TD groups after the 3-day acclimatization period by administration of the synthetic thiamine antagonist pyrithiamine (PYR) and substitution of a thiamine-deficient diet for the regular chow. TD animals were weighed and given a subcutaneous (s.c.) injection of PYR daily. The volume of chow consumed each day was recorded. Control (CON) rats were pair-fed to equal food consumption to that of TD rats using the same thiamine deficient chow and were supplemented with daily s.c. injections of thiamine (THIA). All rats were fed, injected, and assessed for neurological status at the same time each day. Animals in the TD groups were allowed to progress to a state of severe thiamine

deficiency, manifested behaviorally by their loss of righting reflex, but prior to the onset of seizures.

2.2.5.1. Treatment Groups

Rats were randomly assigned to one of the following treatment groups (n=6):

(A) TD/vehicle(VEH) - rats received PYR and saline (1 ml/kg)

(B) TD/DEP - rats received PYR and DEP

(C) TD/MPPE - rats received PYR and MPPE

(D) CON/VEH - rats received THIA and saline. The animals were pair-fed to the TD/VEH Group.

(E) CON/DEP - rats received THIA and DEP. The animals were pair-fed to the TD/DEP Group.

(F) CON/MPPE - rats received THIA and MPPE. The animals were pair-fed to the TD/MPPE Group.

2.2.5.2. Administration of drugs

To avoid route of administration confounds, PYR and THIA were injected s.c., followed immediately by intraperitoneal (i.p.) injections of either VEH, DEP, or MPPE. PYR was administered at 50 μg in 0.2 ml saline per 100 g body weight. THIA was injected at a dose of 10 μg in 0.2 ml saline per 100 g body weight. Animals randomly assigned to receive DEP were administered a daily injection of 1 mg/kg. Rats assigned to

receive MPPE were administered a daily injection at a dose of 0.9 mg/kg (equimolar to 1 mg/kg DEP). Animals designated to receive VEH (1 ml/kg) were administered a daily injection of saline.

2.2.5.3. Reversal Stage Animals

Following the loss of righting reflex, all treatments were discontinued (i.e. TD, VEH, DEP, and MPPE), and all animals received a dose of 10 mg/kg THIA daily for three days, during which time they were fed regular, thiamine-containing chow. The purpose of re-establishing thiamine status was to restore any metabolically damaged neurons. A yoked design was employed to assess the relative potencies of DEP and MPPE as neuroprotective agents. Specifically, when a TD animal was observed to reach the loss of righting reflex stage and its thiamine status reversed, one animal from each of the other treatment groups was also treated with thiamine. After three days of thiamine treatment, the animals were killed by decapitation.

2.2.5.4. Symptomatic Stage Animals

Following the loss of righting reflex, all treatments were discontinued (i.e. TD, VEH, DEP, and MPPE), and animals were sacrificed without restoring thiamine status. Again, a yoked design was employed to assess the relative potencies of DEP and MPPE as neuroprotective agents. Specifically, when a TD animal was observed to reach the loss of

righting reflex stage and was sacrificed, one animal from each of the other treatment groups was also sacrificed.

2.2.6. General sample handling

2.2.6.1. Processing for sectioning

Once the rats were decapitated, the brains were rapidly removed and hemisected along the midline. The right hemisphere was fixed in 10% formalin buffered phosphate in a 50 ml conical for 2 weeks at 4°C to promote even fixation throughout the tissue. Once fixed, brain samples were cut to fit into embedding cassettes for paraffin processing at Dynacare Kasper Laboratories, Edmonton, Alberta. Brain tissue samples were obtained in serial sagittal sections 8 μm thick for use in the study. Anatomical borders based on the atlas of Paxinos and Watson (1982) was used to obtain sections that contained vulnerable areas (e.g. gelatinosa of the thalamus and inferior colliculus). Specifically, several serial sections were obtained of the structures of interest corresponding to Plate 45 (lateral 0.4 mm) and plate 48 (lateral 1.9 mm) of the stereotaxic atlas. Two sections were mounted per positively charged microscope slide and allowed to dry overnight before processing for immunohistochemistry or Nissl stains.

2.2.6.2. Processing for biochemical analyses

The left hemisphere was flash frozen in isopentane on dry ice and stored at –80°C until biochemical analyses. Before the analyses were performed, brain tissue was weighed and 4°C double-distilled water 5x the weight of the sample was added (for a one in six dilution). Using a Teflon® pestle and a glass homogenizing tube, brains were homogenized on ice using a setting of 7 on the motor. Each sample was subjected to 5 strokes of the pestle to ensure consistent homogenization. Aliquots of homogenate (50 µl) were kept at –80°C until MAO activity determination was performed. Analysis of biogenic amines, their acid metabolites, and amino acids were performed immediately after homogenization of the samples.

2.2.6. Nissl Processing

2.2.6.1. Cresyl violet protocol

Sections immediately adjacent to those used for immunohistochemistry were employed for histological evaluation to determine neuronal cell counts by staining with cresyl violet. Cresyl violet is a Nissl stain, which dyes the Nissl substance (endoplasmic reticulum) of cells. This type of stain was used to visualize individual cells, their components, and cellular morphology. Sections were deparaffinized using xylene (3 changes of 5 minutes each) and hydrated to water through graded ethanols from 95%, 90%, to 70% (2 washes of 5 min each). Slides

were then immersed in the cresyl violet for approximately 1 min. Dehydrating the samples through graded ethanols from 70%, 90%, to 95% (2 washes of 5 min each) followed rinsing the slides in tap water. Rinses in xylene (2 changes of 3 min each) cleared the sections of alcohol, and cover slips were applied using Permount®.

2.2.4.2. Neuronal Cell Count Determination

Anatomical borders based on the atlas of Paxinos and Watson (1982) were used to define the various nuclei of interest. Neuronal cell counts were obtained from two brain regions vulnerable to TD, the medial thalamus (gelatinosa; anteromedial nucleus) and the inferior colliculus. In addition, neuronal cell counts were also obtained for the non-vulnerable frontal cortex. Numbers were procured by counting and averaging at least three 1 mm^2 grid areas under x400 magnification for smaller nuclei (e.g. gelatinosa nuclei), and at least six fields for larger nuclei (e.g. inferior colliculus). Criteria for neuronal counting included nuclear size and the presence of a well-defined nucleolus.

2.2.7. Immunohistochemistry

To corroborate neuronal cell loss in the experiments, the use of immunohistochemical techniques was employed. To identify astrocytes, the support cells for neurons, glial fibrillary acidic protein (GFAP) was used. Similarly, the monoclonal antibody ED1 is specific for

macrophage/microglia, which are the principal immune cells of the central nervous system. Thus, increases in GFAP or ED1 immunoreactivity indirectly implies that neuronal injury has occurred.

2.2.7.1. Preparation of phosphate buffered saline (PBS)

Phosphate buffered saline (PBS) used in processing slides for immunohistochemistry consisted of 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 1000 ml of double distilled water at a final pH=7.4. PBS was filtered through 1 micron filter paper, and stored at room temperature until required.

2.2.7.2. Identification of astrocytes

Sections were immunoreacted for GFAP with all incubations performed at room temperature. Slides were deparaffinized using xylene (3 changes of 5 min each) and hydrated to water through graded ethanols from 95%, 90%, to 70% (2 washes of 5 min each). GFAP immunostaining was initiated with a 5 min incubation period in ethanol:H_2O_2 (99:1) to block endogenous peroxidase activity, then rinsed with PBS. To facilitate antigen retrieval, the slides were incubated with Proteinase K for 3 min, followed by PBS rinses. Incubation with Universal Blocker for 20 min was used to eliminate nonspecific background staining. Slides were then incubated with the primary rabbit anti-cow GFAP antibody (1:750 dilution in PBS) in a humid chamber for 60 min, then rinsed with PBS. The next

procedure was incubation with the secondary anti-rabbit immunoglobulin antibody (1:200 dilution in PBS) for 30 min, followed by rinses in PBS. Conjugation with the avidin-biotin complex (1:100) was followed by visualization of immunoreactivity using 3,3'-diaminobenzidine tetrahydrochloride (DAB). Sections were then dehydrated in graded ethanols (from 70%, 90%, to 95%, 2 washes of 5 min each), cleared of ethanol in changes of xylene, and cover slipped using Permount®. Sections incubated without primary antibodies were run as negative controls and showed no immunoreactivity.

2.2.7.3. Microglia Identification

To identify activated microglia/macrophages, ED1 immunostaining was performed similarly to GFAP immunostaining. Briefly, slides were deparaffinized, hydrated, blocked for endogenous peroxide activity, prepared for antigen retrieval, and blocked for non-specific background as described above. Sections were then incubated with monoclonal anti-mouse ED1 (1:500 in PBS) for 60 min in a humid chamber at room temperature, followed by rinses with PBS. The secondary anti-mouse immunoglobulin antibody (1:200 in PBS) was then incubated for 30 min and rinsed with PBS. This was followed by conjugation with avidin-biotin complex (1:100 in PBS). The reaction product was visualized using DAB, and the slides were then dehydrated, cleared, and mounted in

Permount®. Sections incubated without primary antibodies were run as negative controls and showed no immunoreactivity.

2.2.7.4. Quantification of immunoreactivity

Quantification of GFAP and ED1 immunostaining was determined from sections sequential to those used for neuronal cell counts as reported previously (Todd and Butterworth, 1999). A numerical value was assigned to each field based on the percentage of the field labeled and graded as intensity scores (0 = background staining; 1 = <25% of field stained; 2 = 25-50% of field stained; 3 = 50-75% of field stained; 4 = 75-100% of field stained; 5 = entire field darkly stained). The investigator who performed the evaluation of all sections was blind to the treatment groups.

2.2.8. Monoamine oxidase (MAO) activity determination

Determination of brain MAO activity was performed on animals taken to the symptomatic stage as previously described. MAO activity was determined radiochemically by the procedure of Lyles and Callingham (1982), using ^{3}H-5-HT and ^{14}C-β-PEA as specific substrates for MAO-A and MAO-B, respectively.

2.2.8.1. Preparation of potassium phosphate buffer

A 0.2 M solution was required in this assay, and was produced by adding 13.6 g KH_2PO_4 to 500 ml double-distilled water. The pH was adjusted to 7.8 using KOH. The solution was kept at 4°C until required.

2.2.8.2. Radiolabelled substrates

Only 1 μCi/μmol specific activity is required for good resolution in this assay procedure. Stock amine substrates have therefore been diluted with their respective unlabelled amines to give [^{3}H]-5-HT at 50 mM (1 μCi/μmol) and [^{14}C]-PEA at 10mM (1 μCi/μmol) .

2.2.8.3. Preparation of ethyl actetate/toluene

In a volumetric flask that can be fitted with a cap, 400 ml ethyl acetate, 400 ml toluene, and 200 ml double-distilled water were mixed and allowed to sit for at least 24 hours. The top organic layer was then decanted and stored at room temperature until required.

2.2.8.4. Protocol for samples

Brain homogenate (50 μl) was added to 750 μl of phosphate buffer on ice in triplicate. In the blanks, 10 μl 3M HCl was added instead of homogenate, but all other steps were identical. Once mixed, 50 μl of the appropriately labeled substrate was added to the samples ([^{3}H]-5-HT for estimation of MAO-A activity, and [^{14}C]-PEA for estimation of MAO-B activity). The samples were flushed with oxygen and lightly stoppered,

then placed in a water bath held at 37°C for 10 min. The tubes were then removed onto ice and the reaction terminated by the addition of 10 µl 3M HCl. The addition on 1 ml ethyl actetate:toluene (1:1 v/v, water saturated) to extract the radiolabelled products was followed by vortexing twice briefly. The tubes were then centrifuged for 2 min at 1600 rpm, and 700 µl of the organic layer carefully transferred to a scintillation vial. In vials measuring MAO-A activity, 100 µl of glacial acetic acid was added. To all samples, 4 ml of scintillation fluid was added, and the vials were counted for radioactivity.

2.2.9. High performance liquid chromatography (HPLC)

Quantitation of the levels of biogenic amines and their acid metabolites, as well as of amino acids, was performed on the brains of animals taken to the symptomatic stage. Bonded reversed-phase chromatography is the most commonly used type of HPLC (Bowers, 1989), and was the type used in the experiments described in this thesis.

Two methods of mobile phase delivery were employed: the isocratic mode, where composition of the phase remains constant throughout the run, and gradient mode, where the phase composition changes in a stepwise or gradient manner (Bowers, 1989). The isocratic mode was used in the analysis of biogenic amines, and the gradient mode was used in the analysis of amino acids. Two different types of detection

systems were used in the current experiments: fluorescence detection (for amino acids) and electrochemical detection (for biogenic amines).

2.2.9.1. Determination of biogenic amines and their acid metabolites

2.2.9.1.1. Mobile phase preparation

The mobile phase consisted of 920 ml double-distilled H_20, 6.599 g NaH_2PO_4, 197.2 mg SOS, 137.7 mg EDTA, and 80 ml CH_3CN (%). The solution was adjusted to pH = 2.9 with o-phosphoric acid and filtered through 0.25 μm filter paper under vacuum.

2.2.9.1.2. Preparation of perchloric acid solution

In a 250 ml volumetric flask containing 50 ml of double-distilled water, 21.2 ml concentrated (60%) perchloric acid was added carefully. The solution was brought to volume with double-distilled water, and 22 mg of ascorbic acid as well as 250 mg EDTA were added while stirring. The resulting 1 N solution was kept at 4°C until required.

2.2.9.1.3. Protocol for samples

To 450 μL of homogenate, 50 μL of 1 N chilled perchloric acid solution was added to give a final concentration of 0.1 N and vortexed briefly. The samples were then centrifuged on the high speed setting of the microfuge for 2 min and immediately placed on ice. A portion (100 μL)

of the resulting supernatant was transferred into the HPLC plastic inserts and held at 4°C until injected.

Standards for the biogenic amines and their acid metabolites were included in each run. To make solution "A", 10 µl each of NA, DOPAC, DA, 5-HIAA, HVA, and 5-HT (all at 1 mg/ml in double-distilled water) was added to 940 µl 0.1 N perchloric acid solution. A portion of solution "A" (100 µl) was then added to another 900 µl of 0.1 N perchloric acid solution to make solution "B". Six standards of increasing concentrations were then made using solution "B", ranging from 0 to 100 ng, from which a calibration curve was constructed. The flow rate was set at 0.3 ml/min, the column temperature was held at 30°C, and the applied potential of the electrochemical detector was set at 0.85 volt. The run time was approximately 60 min/sample.

2.2.9.2. Determination of amino acids

2.2.9.2.1. Preparation of mobile phases

Mobile phase A contained 900 ml double-distilled water, 8.7548 g NaH_2PO_4, 240 ml methanol (MeOH), 20 ml acetonitrile, and 10 ml tetrahydrofuran (THF; stored under N_2). Before use, it was degassed under vacuum through a 0.25 µm filter paper.

Mobile phase B included 1340 ml double-distilled water, 6.566 g NaH_2PO_4, 1110 ml MeOH, and 60 ml THF. This solution was adjusted to

pH of 6.2 with 10 N NaOH, and degassed under vacuum through a 0.25 μm filter paper before use.

2.2.9.2.2. *Protocol for samples*

To 400 μl MeOH on ice, 100 μl of homogenate was added and mixed by hand with a small pestle. This first mixture was then centrifuged for 2 min at the high speed setting in the microfuge, and 50 μl of the supernatant was combined with another 450 μl MeOH on ice. This second mixture was briefly vortexed and centrifuged on the high speed setting for 2 min. Aliquots (100 μl) of the resulting supernatant were transferred into HPLC inserts and held at 4°C until analysis.

Standards for amino acids were included in each run. To prepare solution "A", stock solutions of amino acids (1 mg/ml in double-distilled water) were added to 866 μl of a 20% MeOH solution. The volumes of amino acids added were: 40 μl glutatmate (GLU); 20 μl each of γ-amino-butyric acid (GABA), serine (SER), and glutamine (GLN); 10 μl each of aspartate (ASP) and taurine (TAUR); 5 μl each of glycine (GLY) and alanine (ALA); and 2 μl asparagine (ASN) and β-alanine (β-ALA). Six standards of increasing concentration of solution "A" was then made, ranging from 0 to 5 μg, from which a calibration curve was constructed.

The gradient mode of mobile phase delivery was employed for analysis of amino acids. The initial gradient was 60% mobile phase A with 40% mobile phase B at a flow rate of 0.5 ml/min. After 10 min, the

gradient was changed to 100% mobile phase B. At 12 min, the flow rate was increased to 0.7 ml/min, and at 20 min the flow rate was further increased to 1.0 ml/min. Finally, at 40 min, the flow rate and gradient were returned immediately to the initial conditions to run for at least 20 min to condition the column.

The column temperature was set at 30°C, and the fluorescence detector was set for an excitation wavelength of 260 nm and an emission wavelength of 455 nm. The o-phthaldialdehyde (OPA) stock was prepared daily. For each sample, standard, or blank to be analyzed, 5 µl of OPA reagent was added to it and the mixture held in the loop for 1.5 min before injecting.

2.3. STATISTICAL ANALYSIS

The program used for data analysis and graph construction was GraphPad™ Prism 3.0 (San Diego, CA). Results from experiments of neuronal cell counts and rat brain homogenates were compared using one-way analysis of variance, and where significant main effects were achieved, *post hoc* Newman Keuls multiple comparisons were performed to ascertain significantly different groups. For GFAP and ED1 immunoreactivity scores, the non-parametric Kruskal-Wallis test was applied to the data. MAO activities were expressed as percent of CON/VEH activity. Values on graphs represent the means ± SEM. A probability of $p<0.05$ was chosen to represent statistical significance.

CHAPTER 3 Results

3.1. BEHAVIOR

All TD/VEH rats exhibited a reduction in food intake after 9 - 10 days of treatment with a concomitant decrease in body weight. During this time period, grooming behavior began to deteriorate. After 11 - 13 days of treatment, these animals displayed increased sensitivity to touch and sound, followed by lethargy, mild ataxia, exopthalmos, and opisthotonos. Symptoms gradually worsened over the subsequent 24 hours, and progressed to a loss of righting reflex by day 14 – 15. In contrast, none of the TD/DEP animals exhibited overt neurological symptoms; only one TD/MPPE rat presented with neurological symptoms at the same time period as the TD/VEH animals.

3.2. HISTOLOGY AND IMMUNOHISTOCHEMISTRY

3.2.1. Neuronal cell counts

Neuronal cell counts obtained from the experimental groups are displayed in Table I. Because there were no significant differences in cell counts obtained from any of the obligate control groups ($p>0.05$), only counts of the CON/VEH group are presented, and these are referred to as controls in Table 3.1. The * indicates the group is significantly different from the control group ($p<0.05$).

In the DEP- and MPPE- treated TD animals, neurons in each of the nuclei assessed appeared to be of normal size and shape, and were of

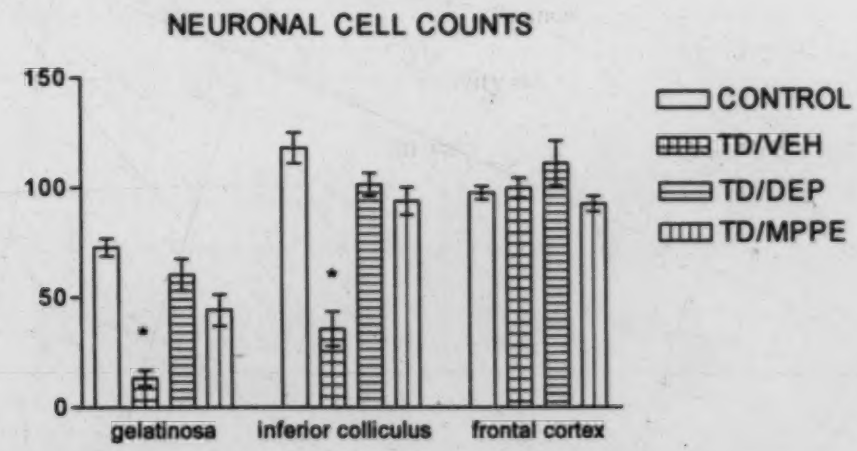
comparable density to those observed in the same nuclei of CON rats. In contrast, the vulnerable regions of the VEH-treated TD rats contained significantly fewer neurons. In sections obtained from these animals, many surviving neurons were shrunken and triangular in shape. In the thalamus and inferior colliculus of the MPPE- and VEH- treated TD rats, relatively small, round, and densely Nissl-stained cells (assumed to be astrocytes) were observed.

Table 3.1. Neuronal cell counts

Brain region	Treatment			
	control	TD/DEP	TD/MPPE	TD/VEH
Gelatinosa (thalamus)	72.8± 2.4	60.3±7.3	44.2±7.1	13.5±3.4*
Inferior Colliculus	118.0±7.0	101.3±5.2	93.7±6.2	35.6±7.8*
Frontal Cortex	97.5±3.0	111±10.3	92.6±3.5	99.8±4.5

Results are expressed as means ± SEM, n=6. * = significantly different from control group.

Figure 3.1. Graphical representation of neuronal cell counts.



Results are expressed as means ± SEM, n=6. * = significantly different from control group.

3.2.2. Immunoreactivity

Intensity scores for GFAP and ED1 immunoreactivity obtained from the experimental groups are displayed in Tables 3.2 and 3.3, respectively. Because there were no significant differences in cell counts obtained from any of the obligate control groups ($p>0.05$), only counts of the CON/VEH group are presented, and these are referred to as controls in the tables. The * indicates a significant difference from the control group ($p<0.05$).

The only group to show a significant increase in GFAP intensity score relative to the control group was the VEH-treated TD group. Specifically, in sections obtained from all vulnerable brain regions of TD/VEH rats, an intense area of GFAP-immunopositive cells surrounding a central area devoid of staining was observed (Figure 3.5.). No significant differences were evident in rats treated with DEP or MPPE in any of the regions examined.

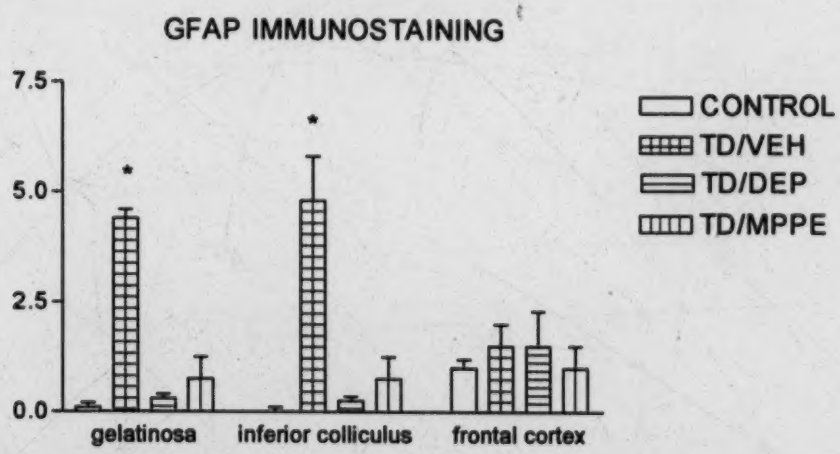
To elucidate the nature of this area, sequential sections were immunoreacted with the microglial marker ED1. This immunostaining revealed that the central core region was intensely immunopositive for ED1 (Figure 3.5.). In the gelatinosa and inferior colliculus, there was a significant increase in ED1 intensity scores for VEH-treated rats compared to control rats. In sections obtained from control, TD/DEP, and TD/MPPE animals, less than 1% of all fields assessed were immunopositive for ED1.

Table 3.2. GFAP Intensity scores

Brain region	Treatment			
	control	TD/DEP	TD/MPPE	TD/VEH
Gelatinosa (thalamus)	0.1±0.1	0.3±0.1	0.75±0.5	4.4±0.2*
Inferior Colliculus	0.0±0.1	0.25±0.1	0.75±0.5	4.8±1.0*
Frontal Cortex	1.0±0.2	1.5±0.8	1.0±0.5	1.5±0.5

Results are expressed as means ± SEM, n=6. * = significantly different from control group. Units are described in the text.

Figure 3.2. Graphical representation of GFAP immunostaining.



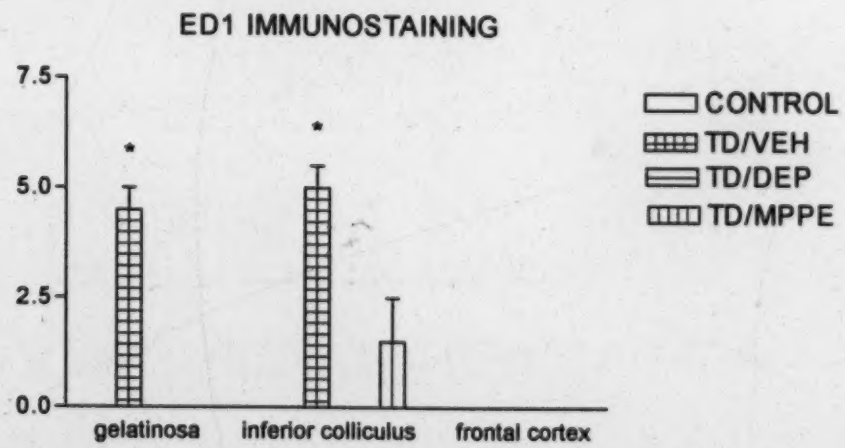
Results are expressed as means ± SEM, n=6. * = significantly different from control group. Units are described in the text.

Table 3.3. ED1 Intensity scores

Brain region	Treatment			
	control	DEP	MPPE	VEH
Gelatinosa (thalamus)	0.0±0.0	0.0±0.0	0.0±0.0	4.5±0.5*
Inferior Colliculus	0.0±0.0	0.0±0.0	1.5±1.0	5.0±0.5*
Frontal Cortex	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Results are expressed as means ± SEM, n=6. * = significantly different from control group. Units are described in the text.

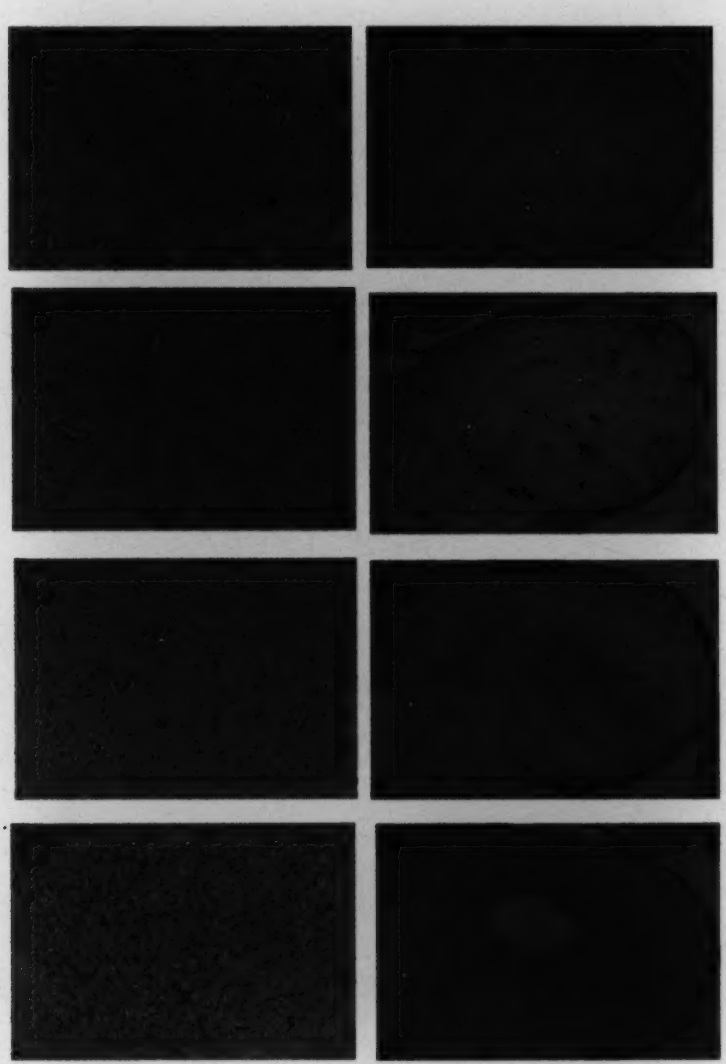
Figure 3.3. Graphical representation of ED1 immunostaining.



Results are expressed as means ± SEM, n=6. * = significantly different from control group. Units are described in the text.

Photomicrographs of representative sections obtained from the inferior colliculus of control (A & E), TD/DEP (B & F), TD/MPPE (C & G), and TD/VEH (D & H) animals are presented in Figure 3.4. Plates A – D display cresyl violet-stained sections (100x); plates E – H display GFAP-immunoreacted sections (40x). A significant decrease in the number of neurons in the section obtained from a TD/VEH (D) rat is evident compared to those obtained from control (A), TD/DEP (B), and TD/MPPE (C) rats. Sections obtained from control, TD/DEP, and TD/MPPE rats exhibit a similar density of neurons, with a compact neuropil. Identification of reactive astrocytes by GFAP immunoreactivity demonstrates that sections obtained from TD/VEH rats (H) exhibited increased immunopositive cells compared to the drug-treated and control groups (E, F, G).

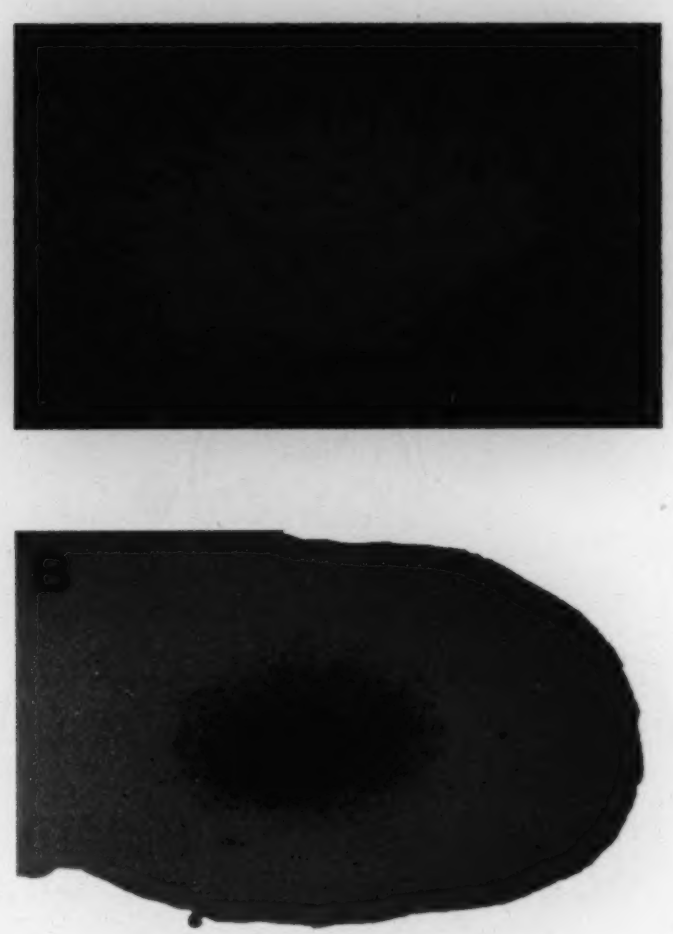
Figure 3.4. Photomicrographs of histology and immunohistochemistry



Photomicrographs of representative sections obtained from the inferior colliculus of control (A&E), TD/DEP (B&F), TD/MPPE (C&G), and TD/VEH (D&H) animals. Plates A-D display cresyl violet-stained sections (100x); plates E-H display GFAP-immunoreacted sections (40x)

Photomicrographs of representative sections obtained from the inferior colliculus of TD/VEH animals are presented in Figure 3.5. Plate A displays a GFAP-immunoreacted section (100x); plate B displays a sequential section immunoreacted with ED1 (40x).

Figure 3.5 Photomicrographs of GFAP and ED1 immunoreactivity



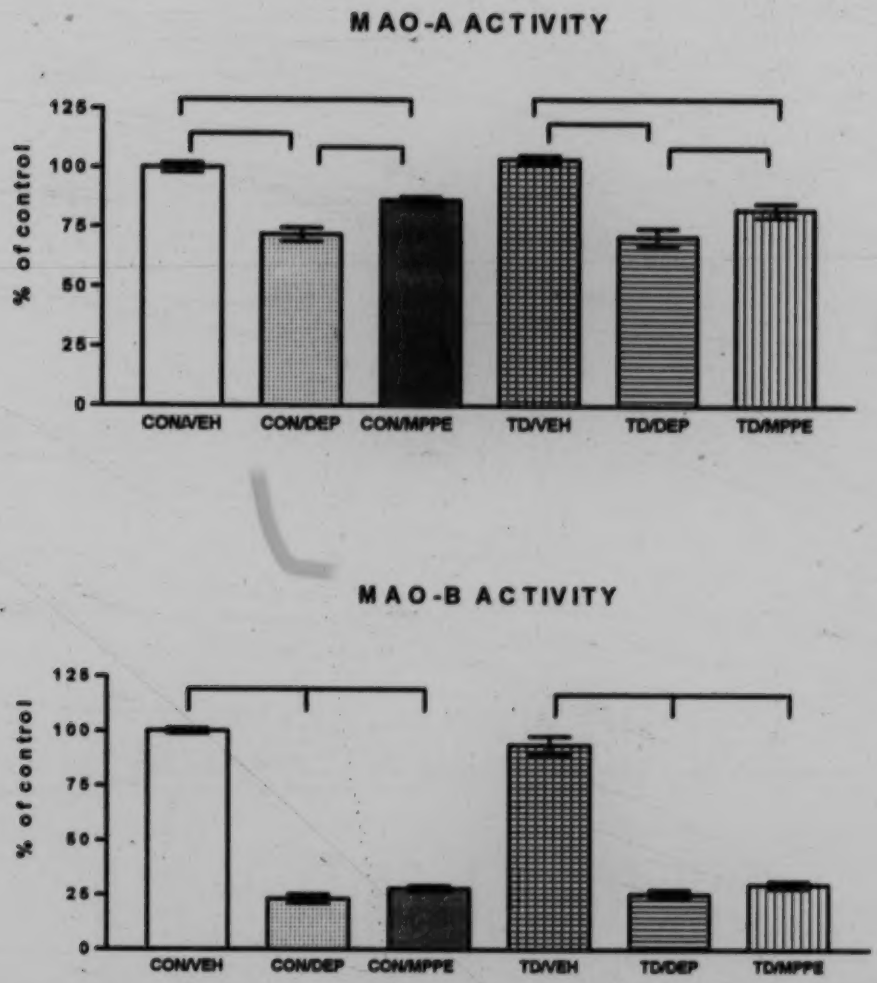
Photomicrographs of representative sections obtained from the inferior colliculus of TD/VEH animals. Plate A displays a GFAP-immunoreacted section (100x); Plate B displays an ED1-immunoreacted section (40x)

3.3. MAO ACTIVITY

MAO-A and MAO-B activities obtained from the experimental groups are displayed in Figure 3.6. Results are expressed as percent of the CON/VEH group. Brackets indicate groups that are significantly different from each other ($p<0.05$).

After 14-15 days of treatment with DEP or MPPE, significant inhibition of MAO-A activity (71.7±2.7[SEM]% and 86.3±1.5% of CON/VEH values, respectively) was observed. The activity of MAO-B was effectively inhibited by both DEP (23.1±2.0% of CON/VEH) and MPPE (27.8±1.4% of CON/VEH), with the extent of inhibition being comparable between the drugs. Concomitant treatment with pyrithiamine or thiamine did not alter the degree of MAO-A or MAO-B inhibition by either DEP or MPPE.

Figure 3.6. MAO activity



Results are expressed as percent of CON/VEH ± SEM, n=6. Brackets indicate significant differences between groups.

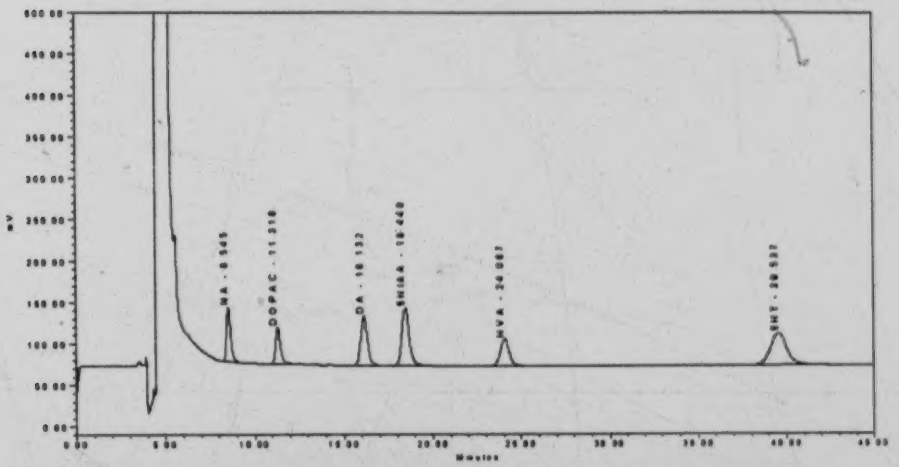
3.4. LEVELS OF BIOGENIC AMINES AND THEIR ACID METABOLITES

Representative chromatograms of a standard and a CON/VEH sample are shown in Figure 3.7. Levels of biogenic amines and their acid metabolites in the experimental groups for animals taken to the symptomatic stage are shown in Figures 3.8. and 3.9., respectively. Brackets indicate groups that are significantly different from each other ($p<0.05$).

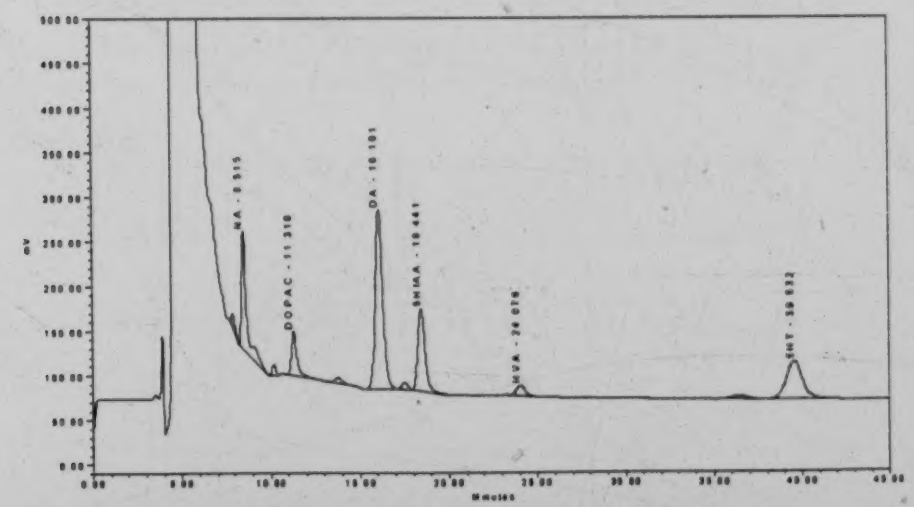
Statistical analyses of levels of biogenic amines and their acid metabolites show that in control animals, DEP and MPPE treatments similarly increased dopamine (DA) levels (550.0±15.2 [SEM] and 545.1±9.7 ng/g tissue, respectively, vs. 413.5±62.3 ng/g tissue in CON/VEH animals). Likewise, drug treatments also increased serotonin (5HT) levels (213.4±6.1 and 201.9±8.7 ng/g tissue, respectively) compared to CON/VEH animals (148.9±5.71 ng/g tissue). Under conditions of TD, DEP treatment significantly increased DA levels relative to TD/VEH rats (629.9±9.9 ng/g tissue vs. 536.0±17.0 ng/g tissue), but MPPE treatment did not. Interestingly, in TD animals treated with VEH, although DA levels were similar to the drug-treated animals, the levels of the metabolite DOPAC were significantly increased compared to all other groups ($p<0.05$). Similarly, levels of HVA were also significantly increased over all other groups, except for the CON/VEH group. Other biogenic

amines and acid metabolites analyzed (NA and 5-HIAA) did not demonstrate any significant differences between groups (data not shown).

Figure 3.7. Chromatograms of biogenic amines and acid metabolites

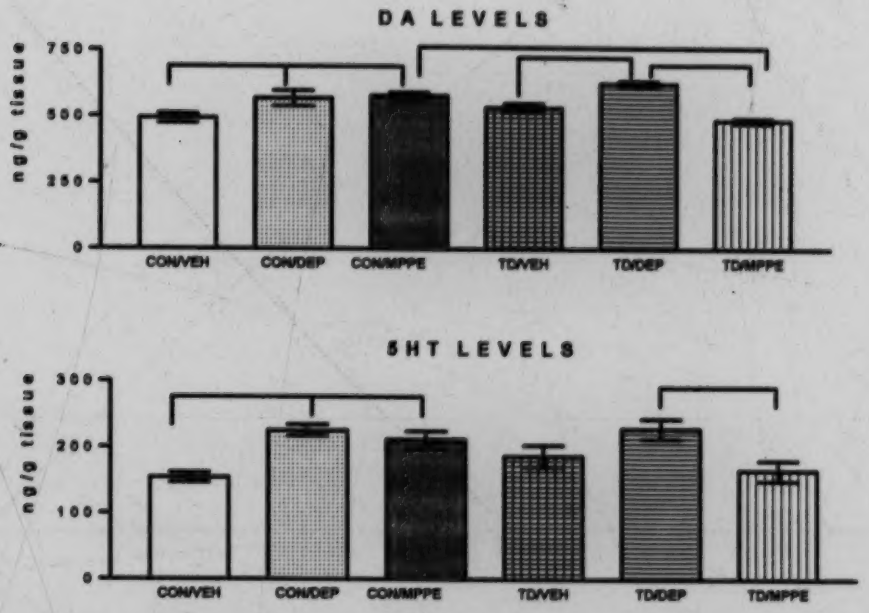


Typical HPLC trace obtained in analysis of biogenic amines and their metabolites in a standard sample. NA, noradrenaline; DOPAC, 3,4-dihydorxyphenylacetic acid; DA, dopamine; 5-HIAA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; 5-HT, 5-hydroxytryptamine, serotonin.



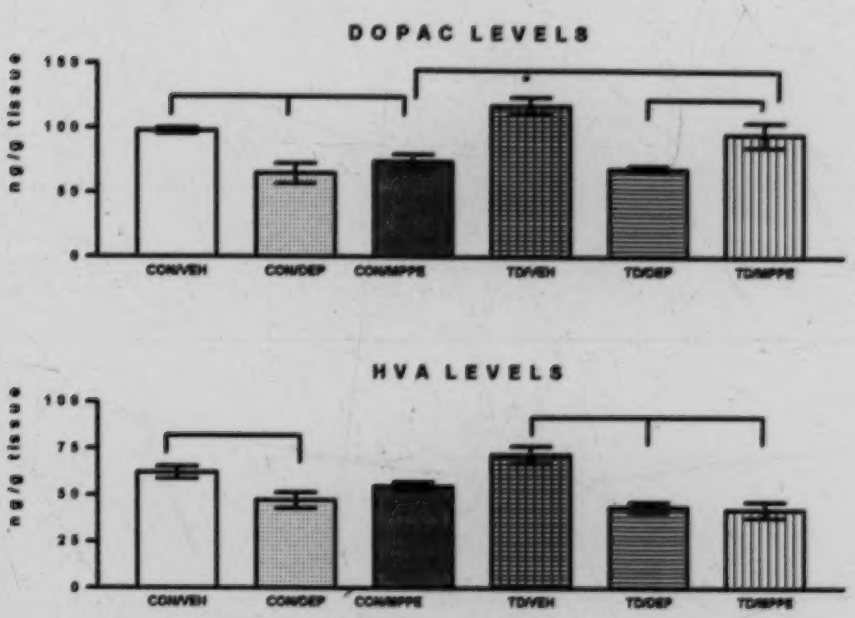
Typical HPLC trace obtained in analysis of biogenic amines and their metabolites in a CON/VEH sample. NA, noradrenaline; DOPAC, 3,4-dihydorxyphenylacetic acid; DA, dopamine; 5-HIAA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; 5-HT, 5-hydroxytryptamine, serotonin.

Figure 3.8. Levels of DA and 5-HT.



Results are expressed as means ± SEM, n=6. Brackets indicate significant differences between groups.

Figure 3.9. Levels of DOPAC and HVA.



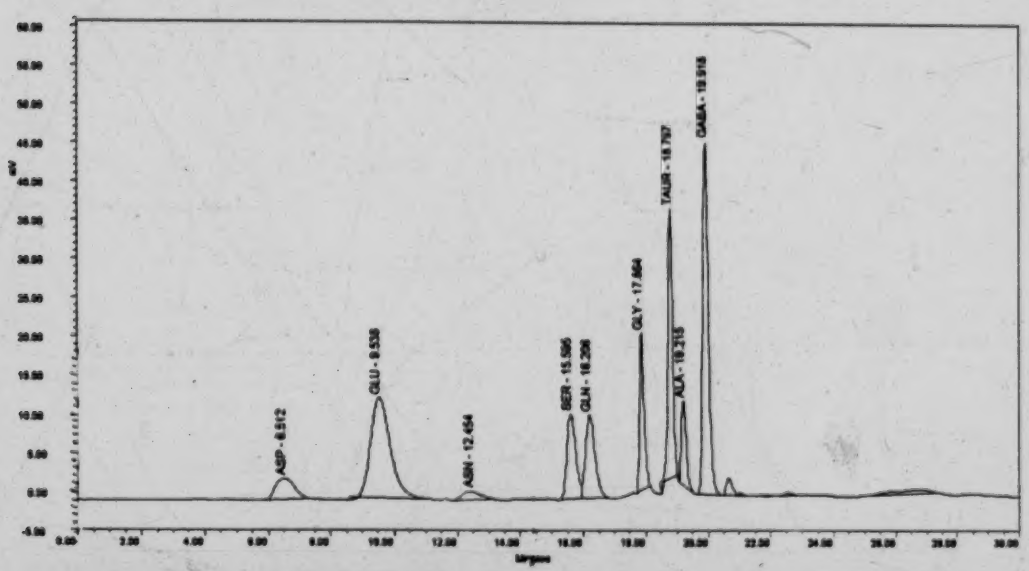
Results are expressed as means ± SEM, n=6. Brackets indicate significant differences between groups. * = significantly different from all other groups.

3.5. LEVELS OF AMINO ACIDS

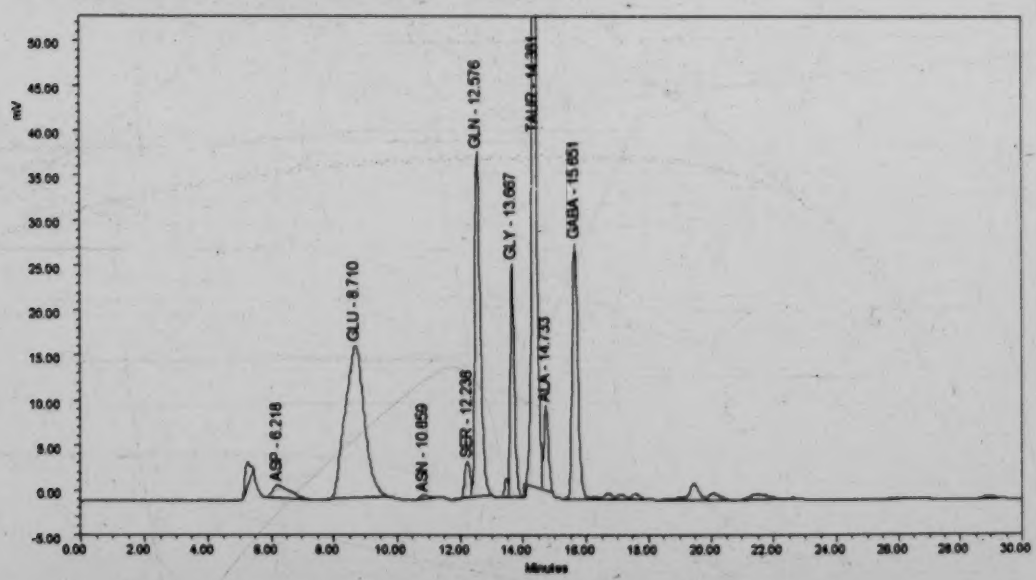
Representative chromatograms of a standard and a CON/VEH sample are shown in Figure 3.10. HPLC analysis of amino acid levels for experimental groups are shown in Figures 3.11. and 3.12. Brackets indicate groups that are significantly different from each other ($p<0.05$).

Statistical analysis demonstrated that TD rats treated with VEH had significantly reduced glutamate and aspartate levels, and significantly increased alanine levels compared to all other groups. TD rats treated with DEP and MPPE had glutamate and alanine levels similar to those obtained from CON animals. Levels of aspartate for DEP- and MPPE-treated TD rats were increased relative to VEH-treated TD rats (604.3±54.6 and 489.9±50.8 μg/g tissue, repectively, vs. 299.6±14.5 μg/g tissue). Glutamine levels in TD/VEH animals were significantly increased compared to CON animals (945.5±25.2 vs. 709.8±15.6 μg/g tissue), while TD rats treated with DEP had glutamine levels comparable to those of CON rats. Similarly, the level of taurine in TD/VEH animals was significantly decreased compared to CON animals (658.7±20.3 μg/g tissue vs. 767.2±16.65 μg/g tissue), while TD rats treated with DEP and MPPE had taurine levels comparable to those of CON rats. All other amino acids (GABA, glycine, serine, and asparagine) analyzed showed no significant differences between groups (data not shown).

Figure 3.9. Chromatograms of amino acids.

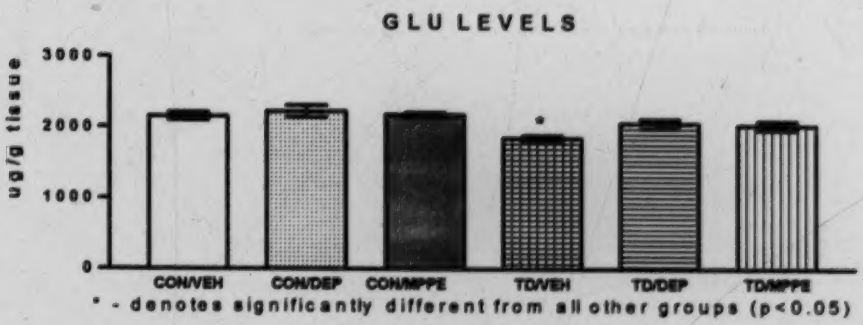


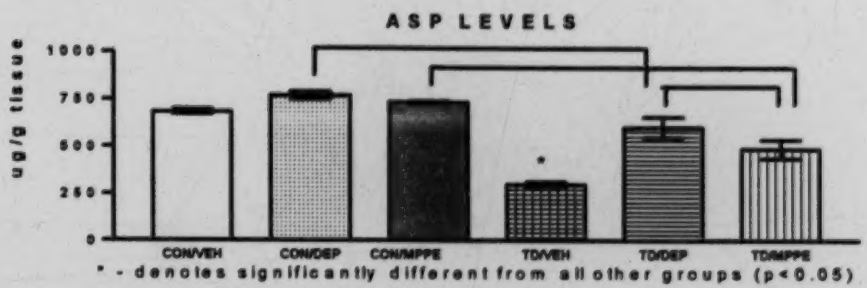
Typical trace obtained in analysis of amino acids in a standard sample. ASP, aspartic acid; GLU, glutamate; ASN, aparagine; SER, serine; GLN, glutamine; GLY, glycine; TAUR, taurine; ALA, alanine; GABA, gamma-aminobutyric acid.

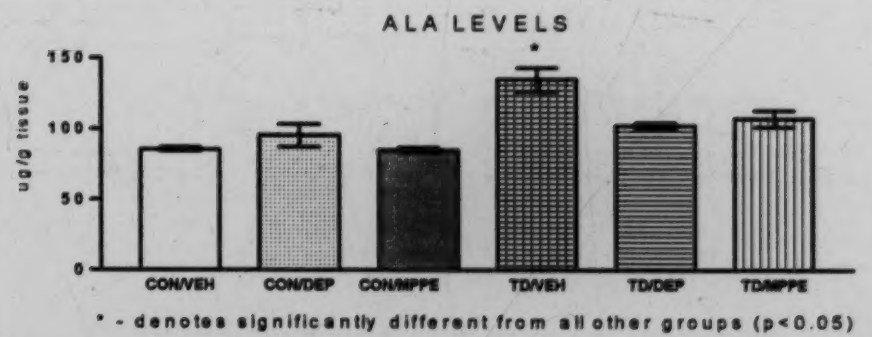


Typical trace obtained in analysis of amino acids in a CON/VEH sample. ASP, aspartic acid; GLU, glutamate; ASN, aparagine; SER, serine; GLN, glutamine; GLY, glycine; TAUR, taurine; ALA, alanine; GABA, gamma-aminobutyric acid.

Figure 3.11. Levels of GLU, ASP, and ALA.

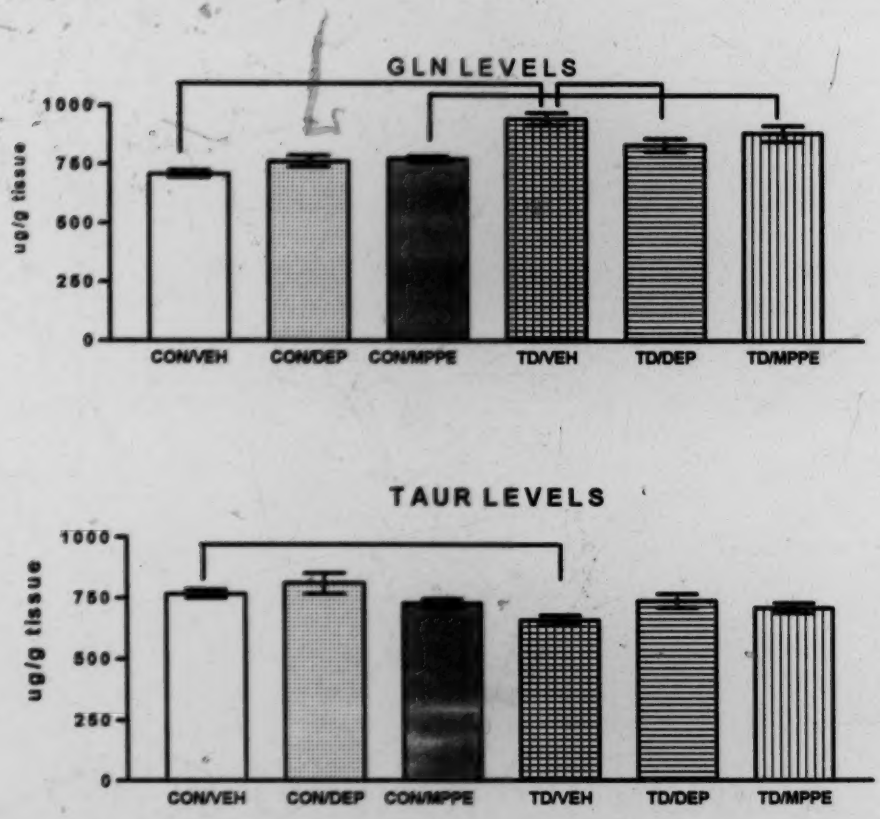






Results are expressed as means ± SEM, n=6. Brackets indicate significant differences between groups. * = significantly different from all other groups.

Figure 3.12. Levels of GLN and TAUR.



Results are expressed as means ± SEM, n=6. Brackets indicate significant differences between groups.

CHAPTER 4 Discussion

4.1. SUMMARY AND IMPLICATIONS

Results of the present studies reveal that the DEP analogue, MPPE, offers significant neuroprotection against neuronal cell loss induced by thiamine deficiency. Histological analyses of tissue sections obtained from the thiamine-deficient treatment groups showed significantly increased neuronal cell survival in the thalamus and inferior colliculus of MPPE-treated rats compared with VEH-treated rats. Confirming previous reports, DEP treatment protected against neuronal loss in brain regions vulnerable to TD (Todd and Butterworth, 1998a). Both MPPE- and DEP-treated rats showed morphologically normal neurons in areas vulnerable to TD, whereas VEH-treated rats possessed shrunken and ischemic-like neurons. Adjacent sections to those stained for neuronal cell counts were GFAP immunoreacted as a measure of astrocytic proliferation, and ED1 antibodies were employed for the identification of activated microglia/macrophages, the principal immune cells in the central nervous system (Carson and Sutcliffe, 1999). Sections obtained from TD/VEH-treated rats showed significantly more GFAP-positive astrocytes as compared to control rats. The intense immunoreactivity observed in TD/VEH animals was not observed in TD/DEP or TD/MPPE animals; however, the TD/MPPE rats did exhibit a slight, but not significant increase in GFAP immunostaining. The effect of DEP and MPPE treatments on GFAP immunoreactivity may be explained by the ability of

these drugs to protect neurons from oxidative stress, consequently attenuating the infiltration of activated astrocytes observed in VEH-treated TD animals (Todd and Butterworth, 1998a). Similarly, in sections from TD/VEH rats, significantly more ED1-positive microglia/macrophages were observed relative to those from control rats. Both DEP- and MPPE-treated TD rats displayed very little ED1-immunostaining, similar to that observed in the control rats.

Analysis of the effects of DEP and MPPE on the activity of MAO-A and –B in the treatment groups showed that both MPPE and DEP treatment resulted in inhibition of both MAO-A and –B activity. The percent of MAO-B inhibited by DEP and MPPE was similar in both normal and thiamine-deficient rats. In contrast, chronic treatment of DEP resulted in significantly greater inhibition of MAO-A than did chronic treatment of MPPE. Thus, at the dose and time interval used, MPPE appears to be more selective than DEP at inhibiting MAO-B over MAO-A. This may have clinical significance, as one of the limitations of treatments using MAO inhibitors is the potential of serious interactions with tyramine-containing foods due to the inhibition of MAO-A (Blackwell et al., 1967). DEP may cause considerable inhibition of MAO-A after chronic use or at increased doses (Gerlach et al., 1992); the results observed in the present studies suggest that this could be less of a problem with MPPE. However, the neuroprotective effects of DEP and MPPE are unlikely to be due to inhibition of MAO (for review, see Tatton and Chalmers-Redman, 1996).

With respect to brain levels of biogenic amines and their acid metabolites, MPPE and DEP treatment exerted similar effects. As expected, in control animals, DEP treatment induced an increase in the level of DA and a decrease in the levels of DOPAC and HVA relative to VEH-treated animals. MPPE treatment also increased the level of DA, with a concomitant decrease in DOPAC level to the same magnitude as DEP treatment. However, in thiamine-deficient animals, the effects on levels of DA were observed only in the DEP-treated group. This result is curious, as both MPPE and DEP had comparable levels of MAO B inhibition in control and thiamine deficient groups. However, in rodents, both MAO-A and MAO-B metabolize DA with equal affinity (Green et al., 1977), which may explain the differences observed between DEP and MPPE treatment under conditions of TD with respect to DA and its acid metabolite levels. The metabolism of DEP into pharmacologically active metabolites (i.e. AM, METH, and dmDEP; Reynolds et al., 1978; Mytilineou et al., 1998) may also be involved in the observed differences between the thiamine-deficient drug treated groups. Interestingly, VEH-treated TD animals displayed increased levels of DA metabolites, yet the level of DA was similar to that of drug-treated controls. This finding offers support for the role of oxidative stress in the pathogenesis of lesions induced by TD, as one of the major causes of superoxide radical formation in vivo is the catabolism of catecholamines (Graham, 1984).

Confirming previous reports, TD/VEH rats displayed increased 5HIAA levels (Van Woert et al., 1979; Langlais et al., 1987) relative to their pair-fed controls (data not shown). In the present study, measurements revealed an increase in 5HT level in DEP-treated rats relative to VEH-treated rats in both control and thiamine-deficient conditions. However, this increase in 5HT did not occur with a concomitant change in 5HIAA levels in the TD/DEP rats. In MPPE-treated animals, the levels of 5HT and 5HIAA were equivalent to those observed for DEP-treated rats under control conditions. However, under conditions of thiamine deficiency, no significant differences were found in measurements of 5HT or 5HIAA in MPPE-treated rats relative to VEH-treated animals. The effect on 5HT and 5HIAA under conditions of TD may be due to the finding that MPPE inhibition of MAO-A, which preferentially uses 5HT as a substrate (Yu, 1986), was less than that of DEP. It has been suggested that TD induces increases in 5HT metabolism and/or impairment of 5HIAA efflux from the brain (Plaitakis et al., 1978; Mousseau et al., 1996). The results from the present studies are in agreement with this theory. These data also suggest that DEP and MPPE treatment restores the altered brain levels of biogenic amines induced by TD to those observed in non-brain damaged control animals.

Quantitation of the amino acids in all of the treatment groups showed that TD VEH-treated rats had significantly reduced glutamate and aspartate levels, and significantly increased alanine levels compared to all

other groups. These results are expected due to the effect of TD on thiamine-dependent enzymes, and confirmed previous findings (Plaitakis et al., 1979; Butterworth and Heroux, 1989). The decreases observed in glutamate and aspartate could result from diminished synthesis of these amino acids due to a TD-induced decrease in αKGDH activity. Similarly, the increase in alanine is consistent with diminished cerebral pyruvate oxidation. In support of this, administration of thiamine to symptomatic TD animals reverses these amino acid levels to those of controls (data not shown) and almost completely restores αKGDH activity (Butterworth and Heroux, 1989). These changes in amino acid levels are also correlated with significant improvement in clinical symptoms (Plaitakis et al., 1979). TD rats treated with DEP or MPPE had glutamate, alanine, and taurine levels comparable to those of CON animals, i.e. there was a trend for aberrations in amino acid levels induced by TD to be counteracted by DEP and MPPE treatment. Thus, DEP and MPPE may protect neurons by preventing aberrations in thiamine-dependent enzymes induced by TD through some unknown mechanism, which maintains normal amino acid levels and energy utilization, resulting in increased neuronal survival.

DEP is metabolized extensively to AM and METH (Heinonen *et al.*, 1989), two compounds which have become widely misused as psychostimulatory and hallucinatory agents, demonstrating their deleterious effects on behavior. As well, there is increasing evidence that suggests that AM-like substances are potentially neurotoxic. For example,

it has been shown that AM is toxic *in vivo* to dopaminergic neurons and that METH is toxic to both dopaminergic and serotonergic neurons (Ricaurte *et al.*, 1985; Ricaurte *et al.*, 1992; Fumagalli *et al.*, 1998). More recently, Stumm *et al.* (1999) demonstrated that these compounds induce apoptosis in rat neocortical neurons *in vitro*. Therefore, the use of DEP in the treatment of neurodegenerative disorders may be limited by its metabolism into AM and METH, compounds whose effects may oppose the neuroprotective properties of DEP.

Given the possible adverse effects of DEP, the development of DEP analogues that are not metabolized into AM-like compounds may provide insight into the mechanisms by which DEP affords neuroprotection. The novel DEP analogue, MPPE, would not be metabolized to AM or METH, yet is still able to protect neurons from thiamine deficiency. Other DEP analogues that are not metabolized to amphetamines (e.g. aliphatic N-propargylamines) have also demonstrated neuroprotective efficacy against neurotoxins (Yu *et al.*, 1992; Yu *et al.*, 1994). These reports and the present results suggest that the AM metabolites of DEP are not necessary for promoting neuronal survival. In addition, it has recently been shown that the DEP metabolite, dmDEP, also possesses neuroprotective efficacy (Mytilineou et al., 1998), which may factor in the differences seen between DEP and MPPE treatment. DEP, MPPE, and the compounds of Yu *et al.* (1992, 1994) have the

propargylamine moiety in common, implicating this chemical structure as important in the neuroprotective action of DEP.

4.2. LIMITATIONS AND FUTURE DIRECTIONS

The neuroprotective potential of the novel DEP analogue MPPE has been demonstrated in thiamine deficiency-induced neuronal cell death. In the literature, there are many paradigms used to examine the efficacy of DEP as a neuroprotective/neurorescue agent. Some examples of these include hypoxic or ischemic insult, mechanical injury, and the application of biochemical neurotoxins. To clarify the capabilities of MPPE, it should also be assessed for its neuroprotective/neurorescue properties in a variety of models of neuronal cell death. Findings from such analyses may lead to common sites of interaction between DEP and MPPE in the cell death cascade, which may aid in the development of drugs that are specifically targeted to prevent neuronal cell loss.

Several mechanisms of action of DEP have been proposed in the literature. These include: preventing free radical formation and/or increasing antioxidant defenses (Carrillo et al., 1992; Cohen and Spina, 1989; Thiffault et al., 1997); altering gene expression for trophic factors (Seniuk et al., 1994; Riva et al., 1997; Tang et al., 1998); and down-regulating the expression of the transcription factor c-jun, which is directly related to apoptosis (Xu et al., 1999). It would be of interest to assess whether MPPE also produces similar results to DEP in these analyses.

The present findings suggest that these avenues of investigation may elucidate further the mechanisms of action of DEP and similar DEP analogues.

The effect of DEP and MPPE on activities of the thiamine-dependent enzymes may also be investigated. It has been reported that TD induces alterations their functioning (Gibson et al., 1984; Butterworth et al., 1986). DEP and MPPE may restore the changes induced by TD in these enzymes, which may be how these drugs decrease the neuronal cell death due to TD. Specifically, DEP-induced alteration in gene expression may maintain mitochondrial function (Wadia et al., 1998), which has been implicated in the pathogenesis of neuronal death due to TD (Bettendorff et al., 1997; Munujos et al., 1996).

In the current experiments, MPPE treatment inhibited both MAO-A and MAO-B activities; however, MPPE-treated animals had less inhibition of MAO-A than did DEP-treated animals. Thus, MPPE may be more selective at inhibiting MAO-B over MAO-A than DEP. To ascertain the effects on MAO activity, a comprehensive time and dose study is required, as well as experiments to determine the nature of the interaction of MPPE with MAO.

HPLC analyses of biogenic amines, their acid metabolites, and amino acids demonstrated differences in level measurements under conditions of TD, DEP treatment, and MPPE treatment. However, samples were derived from half-brain homogenates, which may dilute the

localized effects of the independent variables. Thus, it may be more meaningful to perform these analyses in the future on discrete regions of the brain that are affected by TD, and on regions that are resistant to cell loss induced by TD under these same treatment conditions. Analyses done in this manner should provide useful information regarding the biochemical changes that occur in vulnerable and non-vulnerable areas. As well, this type of analysis may provide insight into the possible mechanisms of action for the neuroprotective/neurorescue effects of DEP and MPPE, and help to clarify the sequence of events that occurs in the cascade of neuronal cell death induced by TD. Furthermore, it remains to be determined whether the changes in biogenic amines and amino acid levels are due to a process in the cell death cascade that is corrected by drug treatment, or whether the drug treatment merely has secondary effects on these levels.

The pattern of immunohistological staining of astrocytes and microglia observed in the vulnerable regions of TD/VEH rats is interesting, and requires further investigation. The present experiments have demonstrated that under conditions of TD, microglial activation is concentrated in the vulnerable brain regions. Further, it has previously been shown that one of the earliest cellular reactions in the brain to TD is microglial activation, which occurs prior to any evidence of neuronal cell death (Todd and Butterworth, 1999). Microglial cells are the brain-resident immune cells (Carson and Sutcliffe, 1999), and, as such, they are the first

cell type to respond to injury by migrating to and proliferating at sites of neuronal damage (Gonzalez-Scarano and Baltuch, 1999). Microglial cells can transform into "activated" microglia, and are a source of potent trophic and cytotoxic molecules, such as cytokines. Cytokines have been implicated in human disorders ranging from cancer to neurodegenerative diseases, as well as trauma and stroke. In addition, iron released from microglia contributes to ROS production, which has been implicated in the cellular events and mechanisms occurring in the development of TD-induced neuronal cell death (Calingasan et al., 1998). Thus, further investigation of the role of microglial activation in the pathogenesis of TD is warranted.

As a variety of events contribute to the pathogenesis of neuronal cell death due to TD, it would be of interest to investigate other, more specific, neuroprotective agents using this model. For example, future studies could investigate the effects of NO on TD-induced neuronal cell death using NOS inhibitors. Other compounds that would be of interest include minocycline (an anti-inflammatory proposed to inhibit microglial activation), cytokine antagonists (e.g. interleukin 1-β antagonist), α-phenyl-N-*tert*-butylnitrone (a nitrone spin trap that reacts covalently with free radicals), GLU receptor antagonists, and GABA-mimetic agents. Experiments that focus on preventing a particular event that occurs in TD may aid in the elucidation its relative importance in the neuronal cell death observed.

4.3. CONCLUSIONS

The TD model is a useful tool for investigating the pathophysiological mechanisms involved in the human condition of WE, as well as selective neuronal cell death in general. This experimental animal model has considerable potential for aiding in the elucidation of the relationship between oxidative deficits, cellular responses, selective neurodegeneration, and the neuroprotective effects of DEP and DEP analogues.

In conclusion, the present investigations demonstrated that the novel DEP analogue, MPPE, affords significant neuroprotection to brain regions vulnerable to thiamine deficiency. This neuroprotection was comparable to that provided by DEP treatment, and animals treated chronically with MPPE showed no adverse behavioral effects or increase in mortality. The effects of MPPE on levels of MAO-B activity were comparable to that of DEP, and chronic treatment with MPPE caused slightly less inhibition of MAO-A than did DEP. Both drugs had similar effects on brain levels of the biogenic amines and amino acids investigated. MPPE may prove to be useful in examining the mechanism of action of DEP and may have potential in the treatment of neurodegenerative disorders.

CHAPTER 5 Bibliography

Aikawa H, Watanabe IS, Furuse T, Iwasaki Y, Satoyoshi E, Sumi T, Moroji T (1984). Low energy levels in thiamine deficiency encephalopathy. J Neuropath Exp Neurol 43:276-287.

Alnermri ES (1997). Mammalian cell death proteases: a family of highly conserved aspartate cysteine proteases. J Cell Biochem 64:33-42.

Au AM, Chan H, Fishman RA (1985). Stimulation of phospholipase A2 activity by oxygen-derived free radicals in isolated brain capillaries. J Cell Biochem 27:449-453.

Bach AWJ, Lan NC, Johnson DL, Abell CW, Bembenek ME, Kwan S-W, Seeburg PH, Shih JC (1988). cDNA cloning of human liver monoamine oxidase A and B: Molecular basis of differences in enzymatic properties. Proc Natl Acad Sci 85:4934-4938.

Banati RB, Gehrmann J, Scheubert P, Kreutzberg GW (1993). Cytotoxicity of microglia. Glia 7:111-118.

Bettendorff L, Goessens G, Sluse FE (1997). Reversibility of thiamine deficiency-induced partial necrosis and mitochondrial uncoupling by addition of thiamine to neuroblastoma cell suspension. Mol Cell Biochem 174:121-124.

Blackwell R, Marley E, Price J (1967). Hypertensive interactions between monoamine oxidase inhibitors and food stuffs. Br J Psychiat 113:329-365.

Bowers LD (1989). Chapter 5: Liquid chromatography in Clinical Chemistry-Theory, analysis, and correlation, 2nd edition (LA Kaplan and AJ Pesce, eds.). p.94-109. The C.V. Mosby Company, St. Louis, Missouri.

Butterworth RF, Giguere J-F, Besnard AM (1986). Activites of thiamine-dependent enzymes in two experimental models of thiamine deficiency encephalopathy. 2. α-ketoglutarate dehydrogenase. Neurochem Res 11:567-577.

Butterworth RF, Heroux M (1989). Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine-dependent enzymes. J Neurochem 52:1079-1084.

Calingasan NY, Chun WJ, Park LCH, Uchida K, Gibson GE (1999). Oxidative stress is associated with region-specific neuronal death during thiamine deficiency. J Neuopathol Exp Neurol 58:946-958.

Calingasan NY, Park LCH, Calo LL, Trifiletti RR, Gandy SE, Gibson GE (1998). Induction of nitric oxide synthase and microglial responses precede selective cell death induced by chronic impairment of oxidative metabolism. Am J Pathol 153:599-610.

Carrillo MC Kanai S Nokubo M Ivy GO Sato Y Kitani K (1992). (-)-Deprenyl increases activities of superoxide dismutase and catalase in striatum but not in hippocampus – the sex and age related differences in the optimal dose in the rat. Exp Neurol 48:517-522.

Carson, M.J. & Sutcliffe, J.G. (1999). Balancing function vs. self defense: The CNS as an active regulator of immune responses. J Neurosci Res 55:1-8.

Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK (1992). Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. J immunol 149:2736-2741.

Cohen G, Spina MB (1989). Deprenyl suppresses the oxidant stress associated with increased dopamine turnover. Ann Neurol 26:689-690.

Cooper JR, Pincus JH (1979). The role of thiamine in nervous tissue. Neurochem Res, 4:223-239.

Cotran RS, Kumar V, Robbins SL (1989). Chapter 1: Cellular injury and adaptation in Robbins Pathological Basis of Disease, 4th ed. (Cotran RS, Kumar V, Robbins SL, eds.). p 1-38. W.B. Saunders Company, Philadelphia, PA.

Darzynkiewicz Z, Bedner E, Traganos F, Murakami T (1998). Critical aspects in the analysis of apoptosis and necrosis. Human Cell 11:3-12.

Dragovich R, Rudin CM, Thompson CB (1998). Signal transduction pathways that regulate cell survival and cell death. Oncogene 17:3207-3213.

Dragunow M, Preston K (1995). The role of inducible transcription factors in apoptotic nerve cell death. Brain Res Rev 21:1-28.

Dreyfus PM (1959). The quantitative histochemical distribution of thiamine in normal rat brain. J Neurochem 4:183-190.

Fang J, Yu PH (1994). Effect of L-deprenyl, its structural analogues and some monoamine oxidase inhibitors on dopamine uptake. Neuropharmacol 33:763-768.

Filip V, Kolibas E. 1999. Selegiline in the treatment of Alzheimer's disease: a long-term randomized placebo-controlled trial. J Psychiatry Neurosci 24:234-43.

Finnegan KT, Skratt JS, Irwin I, DeLanney LE, Langston JW. 1990. Protection against DSP-4-induced neurotoxicity by deprenyl is not related to its inhibition of MAO-B. Eur J Pharmacol 184:119-126.

Fumagalli F, Gainetdinov RR, Valenzano KJ, Caron MG. 1998. Role of dopamine transporter in methamphetamine-induced neurotoxicity: evidence from mice lacking the transporter. J Neurosci 18:4861-4869.

Gelowitz DL, Paterson IA (1999). Neuronal sparing and behavioral effects of the antiapoptotic drug, (-)-deprenyl, following kainic acid administration. Phamacol Biochem Behav 62:225-262.

Gerlach M, Riederer P, Youdim MB (1992). The molecular pharmacology of l-deprenyl. Eur J Pharmacol 226:97-108.

Gibson GE, Ksiezak-Reding J, Sheu K-F R, Mykytyn V, Blass JP (1984). Correlations of enzymatic, metabolic and behavioral deficits in thiamine deficiency and its reversal. Neurochem Res 9:803-814.

Giguere J-F and Butterworth RF (1987). Activities of thiamine-dependent enzymes in two experimental models of thiamine deficiency encephalopathy: 3. Transketolase. Neurochem Res 12:305-310.

Gonzalez-Scarano F and Baltuch G (1999). Microglia as mediators of inflammatory and degenerative diseases. Ann Rev Neurosci 22:219-240.

Graham DG (1984). Catecholamine toxicity: a proposal for the molecular pahtogenesis of manganese neurotoxicity and Parkinson's disease. Neurotoxicology 5:83-95.

Green AR, Michel BD, Tordoff FC, Youdim MBH (1977). The evidence for dopamine deamination by both type A and type B monoamine oxidase in rat brain in vivo and for degree of dopamine and serotonin. Br J Pharmacol 60:505-512.

Grimsby J, Chen K, Wang LJ, Lan LC, Shih JC (1991). Human monoamine oxidase A and B genes exhibit identical exon-intron organization. Proc Natl Acad Sci 88:3637-3641.

Hazell AS, McGahan L, Tetzlaff W, Bedard AM, Robertson GS, Nakabeppu Y, Hakim AM (1998). Immediate-early gene expression in the brain of the thiamine-deficient rat. J Molecular Neurosci 10:1-15.

Hazell AS, Todd KG, Butterworth RF (1998). Mechanisms of neuronal cell death in Wernicke's encephalopathy. Met Brain Dis 13:97-122.

Heinonen EH, Anttila MI, Lammintausta RA (1994). Pharmacokinetic aspects of l-deprenyl (selegiline) and its metabolites. Clin Pharmacol Ther 56:742-749.

Heinonen EH, Myllyla V, Sotaniemi K, Lamintausta R, Salonen J, Anttila M, Savijarvi M, Kotila M, Rinne U (1989). Pharmacokinetics and metabolism of selegiline. Acta Neurol Scand Suppl 126:93-99.

Heroux M, Butterworth RF (1995). Regional alteration of thiamine phosphate esters and of thiamine diphosphate-dependent enzymes in relation to function in experimental Wernicke's encephalopathy. Neurochem Res 20:87–93.

Hutchins JB, Barger SW (1998). Why neurons die:cell death in the nervous system. Anatomical Records 253:79-90.

Iwashima A, Wakabayashi Y, Nose Y (1976). Formation of pyrithiamine pyrophosphate in brain tissue. J Biochem 79:845-847.

Kragten E, Lalande I, Zimmermann K, Roggo S, Schindler P, Muller D, van Oostrum J, Waldmeier P, Furst P (1998). Glyceraldehyde-3-phosphate dehydrogenase, the putative target of the antiapoptotic coumpounds CGP 3466 and R-(-)-deprenyl. J Biol Chem 273:5821-5828.

Lakshmana MK, Rao BSS, Dhingra NK, Ravikumar R, Govindaiah, Sudha S, Meti BL, Raju TR (1998). Role of monoamine oxidase type A and B on the dopamine metabolism in discrete regions of the primate brain. Neurochem Res 23:1031-1037.

Langlais PJ, Anderson G, Guo SX, Bondy SC (1997). Increased cerebral free radical production during thiamine deficiency. Metabolic Brain Disease 12:137-143.

Langlais PJ, Mair RG, Anderson CD, McEntee WJ (1987). Monoamines and metabolites in cortex and subcortical structures: normal regional distribution and the effects of thiamine deficiency in the rat. Brain Res 421:140-149.

Le W, Jankovic J, Xie W, Kong R, Appel SH (1997). (-)-Deprenyl protection of 1-methyl-4 phenylpyridium ion (MPP+)-induced apoptosis independent of MAO-B inhibition. Neurosci Lett 224:197-200.

Leist M, Nicotera P (1998). Apoptosis, excitotoxicity, and neuropathology. Exp Cell Res 239:183-201.

Li X-M, Juorio AV, Qi J, Boulton AA (1998). L-Deprenyl potentiates NGF-induced changes in superoxide dismutase mRNA in PC12 cells. J Neurosci Res 53:235-238.

Li X-M, Qi J, Juorio AV, Boulton AA (1993). Reduction in GFAP mRNA abundance induced by (-)-deprenyl and other monoamine oxidase B inhibitors in C6 glioma cells. J Neurochem 61:1573-1576.

Lyles GA, Callingham BA (1982). *In vitro* and *in vivo* inhibition by benserazide of clorgyline-resistant amine oxidase in rat cardiovascular tissues. Biochem Pharmacol 31: 1417-1424.

Matsushima K, MacManus, JP, Hakim AM (1997). Apoptosis is restricted to the thalamus in thiamine-deficient rats. NeuroReport 8:867-70.

Miwa K, Asano M, Horai R, Iwakure Y, Nagata S, Suda T (1998). Caspase 1-independent Il-1b release and inflammation induced by the apoptosis inducer Fas ligand. Nature Med 4:1287-1292.

Mousseau DD, Rao VL, Butterworth RF (1996). Alteration in serotonin parameters in brain of thiamine-deficient rats are evident prior to the appearance of neurological symptoms. J Neurochem 67:1113-1123.

Munujos P, Coll-Canti J, Beleta J, Gonzalez-Sastre F, Gella FJ (1996). Brain pyruvate oxidation in experimental thiamin-deficiency encephalopathy. Clin Chim Acta 255:13-26.

Mytilineou C, Leonardi E, Radcliffe P, Heinonen EH, Han S, Werner P, Cohen G, Olanow CW (1998). Deprenyl and desmethylselegiline protect mesencephalic neurons from toxicity induced by glutathione depletion. J Pharmacol Exp Ther 284:700-706.

Nakajima, K. & Kohsaka, S. (1998). Functional roles of microglia in the central nervous system. Human Cell 11:141-155.

Oppenheim RW (1991). Cell death during development of the nervous system. Ann Rev Neurosci 14:453-501.

Parkinson Study Group (1989). Effect of deprenyl on the progression of disability in early Parkinson's Disease. N Engl J Med 321:1364-1371.

Parkinson Study Group (1993). Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's Disease. N Engl J Med 328:176-183.

Paterson IA, Barber AJ, Gelowitz DL, Voll C (1996). (-)-Deprenyl reduces delayed neuronal death of hippocampal pyramidal cells. Neurosci Biobehav Rev 20:1-6.

Paterson IA, Davis BA, Durden DA, Juorio AV, Yu PH, Ivy G, Milgram W, Mendonca A, Wu P, Boulton AA. (1995). Inhibition of MAO-B by (-)-deprenyl alters dopamine metabolism in the macaque (*Macaca fascicularis*) brain. Neurochem Res 20:1503-1510.

Paxinos G, Watson C (1982). The rat brain in stereotaxic coordinates. Academic Press Inc, New York, NY.

Peters RA (1936). The biochemical lesion in vitamin B1 deficiency: application of modern biochemical analyses in its diagnosis. Lancet 230:1161-1165.

Plaitakis A, Nicklas WJ, Berl S (1979). Alterations in uptake and metabolism of aspartate and glutamate in brain of thiamine deficient animals. Brain Res 171:489-502.

Plaitakis A, Van Woert MH, Hwang EC, Berl S (1978). The effect of acute thiamine deficiency on brain tryptophan serotonin and 5-hydroxyindoleacetic acid. J Neurochem 31:1087-1089.

Revuelta M, Venero JL, Machado A, Cano J (1997). Deprenyl induces GFAP immunoreactivity in the intact and injured dopaminergic nigrostriatal system but fails to counteract axotomy-induced degenerative changes. Glia 21:204-206.

Reynolds GP, Elsworth JD, Blau K, Sandler M, Leers AJ, Stern GM (1978). Deprenyl is metabolized to methamphetamine and amphetamine in man. Br J Clin Pharmacol 6:542-544.

Ricaurte GA, Bryan G, Strauss L, Seiden L, Schuster C (1985). Hallucinogenic amphetamine selectively destroys brain serotonin nerve terminals. Science 229:986-988.

Ricaurte GA, McCann UD (1992). Neurotoxic amphetamine analogues: effects in monkeys and implications for humans. Ann N Y Acad Sci 648:371-382.

Rindi G, Perri V (1961). Uptake of pyrithiamine by tissue of rats. Biochem J, 80:214-216.

Riva MA, Molteni R, Racagni G (1997). L-Deprenyl potentiates cAMP-induced elevation of FGF-2 mRNA levels in rat cortical astrocytes. NeuroReport 8:2165-2168.

Salo PT, Tatton WG (1992). Deprenyl reduces the death of motoneurons caused by axotomy. J Neurosci Res 31:394-400.

Sen I and Cooper JR (1976). The turnover of thiamine and its phosphate esters in rat organs. Neurochem Res 1:65-71.

Seniuk NA, Henderson JT, Tatton WG, Roder JC (1994). Increased CNTF gene expression in process-bearing astrocytes following injury is augmented by R(-)-deprenyl. J Neurosci Res 37:278-286.

Sheu K-F R, Calingasan NY, Lindsay JG, Gibson GE (1998). Immunochemical characterization of the deficiency of the α-ketoglutarate dehydrogenase complex in thiamine-deficient rat brain. J Neurochem 70:1143-1150.

Shimazu S, Katsuki H, Akaike A (1999). Deprenyl rescues dopaminergic neurons in organotypic slice cultures of neonatal rat mesencephalon from N-methyl-D-aspartate toxicity. Eur J Pharmacol 377:29-34.

Stumm G, Schlegel J, Schafer T, Wurz C, Mennel HD, Krieg JC, Vedder H (1999). Amphetamines induce apoptosis and regulation of bcl-x splice variants in neocortical neurons. FASEB J 13:1065-1072.

Sunderland T, Tariot PN, Cohen RM, Newhouse PA, Mellow AM, Mueller, EA, Murphy DL (1987). Dose-dependent effects of deprenyl on CSF monoamine metabolites in patients with Alzheimer's disease. Psychopharmacology 91:293-296.

Tang YP, Ma YL, Chao CC, Chen KY, Lee EHY (1998). Enhanced glial cell line-derived neurotrophic factor mRNA expression upon (-)-deprenyl and melatonin treatments. J Neurosci Res 53:593-604.

Tatton WG, Chalmers-Redman (1996). Modulation of gene expression rather than monoamine oxidase inhibition: (-)-Deprenyl-related compounds in controlling neurodegeneration. Neurol 47(suppl3):S171-183.

Tatton WG, Greenwood CE (1991). Rescue of dying neurons: A new action for deprenyl in MPTP Parkinsonism. J Neurosci Res 30:666-672.

Tatton WG Ju WYL Holland DP Tai C Kwan M (1994). (-)-Deprenyl reduces PC12 cell apoptosis by inducing new protein synthesis. J Neurochem 63:1572-1575.

Thiffault C, Quirion R, Poirier J (1997). The effect of L-deprenyl, D-deprenyl and MDL72974 on mitochondrial respiration: a possible mechanism leading to an adaptive increase in superoxide dismutase activity. Mol Brain Res 49:127-136.

Todd KG, Butterworth RF (1997). Evidence that oxidative stress plays a role in neuronal cell death due to thiamine deficiency. J Neurochem 69:5136A.

Todd KG, Butterworth RF (1998a). Increased neuronal cell survival after (-)-deprenyl treatment in experimental thiamine deficiency. J Neurosci Res 52:240-246.

Todd KG, Butterworth RF (1998b). Evaluation of the role of NMDA-mediated excitotoxicity in the selective neuronal loss in experimental Wernicke encephalopathy. Exp Neurol 149:130-138.

Todd KG, Butterworth RF (1999). Early microglial response in experimental thiamine deficiency: an immunohistochemical analysis. Glia 25:190-198.

Todd KG, Hazell AS, Butterworth RF (1999). Alcohol-thiamine interactions: an update on the pathogenesis of Wernicke encephalopathy. Addiction Biol 4:261-272.

Troncoso JC, Johnston MV, Hess KM, Griffin JW, & Price DL (1981). Model of Wernicke's encephalopathy. Arch Neurol 38:350-354.

Van Woert MH, Plaitakis A, Hwang E, Berl S (1979). Effect of thiamine deficiency on brain serotonin turnover. Brain Res 179:103-110.

Wadia JS Chalmers-Reman RME, Ju WJH, Carlile GW, Philips JL, Fraser AD, Tatton WG (1998). Mitochondrial membrane potential and nuclear changes in apoptosis caused by serum and nerve growth factor withdrawal: time course and modification by (-)-deprenyl. J Neurosci 18:932-947.

Wyllie AH, Ker JF, Currie AR (1980). Cell death: the significance of apoptosis. Int Rev Cytol 68:251-306.

Xu L, Ma J, Seigel GM, Ma J-X (1999). L-deprenyl, blocking apoptosis and regulating gene expression in cultured retinal neurons. Biochem Phamacol 58:1183-1190.

Yoshida T, Yamada Y, Yamamoto T, Kuroiwa I (1986). Metabolism of deprenyl, a selective monoamine oxidase (MAO) B inhibitor in rat: relationship of metabolism to MAO-B inhibitory potency. Xenobiotica 16:29-136.

Youdim MBH and Finberg JPM (1991). New directions in monoamine oxidase A and B selective inhibitors and substrates. Biochem Pharmacol 41:155-162.

Yu PH (1986). Monoamine oxidase, in Neuromethods Vol 5: Neurotransmitter enzymes (Boulton AA, Baker GB, and Yu PH, eds). Pp 235-272. Humana Press; Clifton, NJ.

Yu PH, Davis BA, Boulton AA (1992). Aliphatic propargylamines: potent, selective, irreversible monoamine oxidase B inhibitors. J Med Chem 35:3705-3713.

Yu PH, Davis BA, Durden DA, Barber A, Terleckyj I, Nicklas WG, Boulton AA (1994). Neurochemical and neuroprotective effects of some aliphatic propargylamines: new selective nonamphetamine-like monoamine oxidase B inhibitors. J Neurochem 62:697-704.

Zhang X, Zuo D-M, Yu PH (1995). Neuroprotection by R(-)-deprenyl and N-2-hexyl-N-methylpropargylamine on DSP-4, a neurotoxin, induced degeneration of noradrenergic neurons in the rat locus coeruleus. Neurosci Lett 186:45-48.